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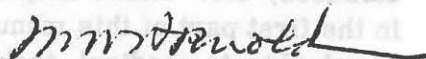
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INTRODUCTION

This manual was prepared by the staff of the Chemistry Branch of the Department of Laboratories of the Naval Medical School to serve as a guide for Laboratory Students and as a working reference for both physicians and technicians in the field of Biochemistry. The procedures and techniques contained herein were selected for their simplicity and accuracy and are in current use in the Laboratories of the Naval Medical School.



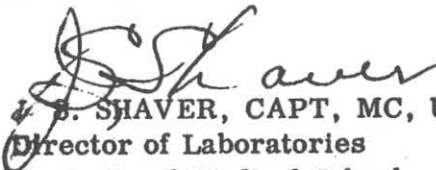
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PREFACE

In recent years clinical pathology has been rapidly expanding, especially in the field of clinical chemistry. Many procedures which once were considered purely research in character, such as transaminase and steroids have been adapted to the routine clinical laboratory. In addition, there have been many refinements and improvements in techniques which have necessitated changes in time-honored methods. To adequately perform laboratory tests it is essential to have a basic understanding of sound laboratory principles, such as primary standards, methods of analysis, chemical balances, etc. These subjects are brought to attention in the first part of this manual and will prove extremely valuable to medical technologists and residents in pathology. The authors are to be commended on the completeness and excellence of this manual on Clinical Chemistry.



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General Chemical Technique

This Manual, in its original form and subsequent revisions, is the result of the combined efforts of both former and present members of the Staff of the Naval Medical School.

"..... most human activities advance by virtue of contributions from many different types of individuals, with vastly different endowments, working at different levels. Medical investigation is no exception to this rule."

Medical Research: A Mid-Century Survey
Boston, Little, Brown and Company, 1955, vol. 1, p. xxx.

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INTRODUCTION

This manual, in general, represents the methods in use at the present time in this laboratory. Some methods in use in the author's laboratory in 1956 (Temple University Hospital, Philadelphia, Pa.) are included as alternate procedures.

These methods derive from many sources, most of which are referred to in the reference section for each procedure. Commonly, the exact procedure is slightly modified to accord with the requirements of this laboratory.

The sections on general principles of chemical analysis will, it is hoped, serve to help to encourage accurate, precise, and knowledgeable work in the laboratory. The material on interpretation should serve as a motivating link between the laboratory worker and the physician in their joint effort directed toward the diagnosis and treatment of disease.

There are, undoubtedly, errors present in this manual, in typography and in text. It is hoped that these will be drawn to our attention so that they may be rectified.

Clinical chemistry seeks by the analysis of biological fluids and tissues to aid the physician in the diagnosis and treatment of disease. The laboratory cannot diagnose but it can aid in diagnosis; the laboratory cannot treat but it can help to guide the physician in his therapeutic efforts. The laboratory is responsible for prompt, rapid and accurate chemical analysis of specimens submitted to it for examination. In many cases, the physician can aid the laboratory by supplying certain information about the patient, such as previous pertinent medical history.

ORGANIZATION AND CONTROL IN THE LABORATORY

A clinical chemistry laboratory should be so staffed and organized that the inevitable errors of technique and of human fallibility will be immediately recognized so that steps may be taken to insure that: (a) the erroneous result will not be sent to the physician and (b) the source of the inaccuracy can be found.

Each individual must be alert to the possibilities of error and when a mistake is detected, must be willing and eager to repeat the determination. A laboratory should never be so under pressure from excessive work load that adequate measures for insuring the requisite degree of accuracy are neglected. It is inevitable that errors in solution preparation, analytical technique and in the calculation of results shall occur; it must be made inevitable that these errors be detected and remedied before they are translated into tragedy for the patient. It can never be as serious for an analyst to admit a mistake as it is for the patient to experience the results of the mistake!

Therefore, it should be evident that the analyst must be completely honest in the obtaining and the calculation of data, not only with his fellow-workers, but also with himself. This is always difficult; the one person easiest to deceive is one's own self. Always remember: your technique may be perfect, the method or solution may be at fault.

In this laboratory, results are never given to any person other than those in immediate charge of the care of the patient: the doctors (the attending physicians) and the nurses. Any others desiring information should secure it from the physician in attendance. Also, the interpretation of results in the laboratory should not be made by the technician except by repeating printed values of normal ranges which are available. Remember--the laboratory cannot diagnose!

The next few paragraphs are taken from "A Curriculum for Schools of Medical Technology" by Israel Davidsohn, M.D., who is Director of Laboratories and Pathologist at Mount Sinai Hospital, Chicago, Illinois.

LABORATORY DISCIPLINE

It should always be remembered that any consideration of medical technology just as of medicine in general must be approached from the viewpoint that the final goal of all work is service to the patient. The patient is the center; everything in medical work—and medical technology is a part of it—revolves around the patient.

This makes it easy to understand the need for discipline in the medical laboratory, which can be likened to a ship on the ocean. Just as the captain of the ship cannot be held responsible for his task unless there is rigid discipline, so must there be discipline in the laboratory if the pathologist is to do well his part of the medical service to the patient.

The technologist must realize the essential need of laboratory discipline; he must be ready and willing to adapt himself to the organization of the laboratory, although it may sometimes entail personal hardship and inconvenience. Those who cannot or will not do it should keep away from medical work of any kind.

THE EMERGENCY IN THE LABORATORY

There are occasions, rather frequent ones, when the life of a patient depends on the results of laboratory tests, on their accuracy, on the speed with which they are performed and on their interpretation. The interpretation is entirely within the scope of activities of the pathologist, as has been stated already. The accuracy of the results

and the speed of performance are two aspects of laboratory work for which the medical technologist is responsible. In his training these two features must always be remembered and emphasized. Medical technologists must be trained in school and long after they have completed their undergraduate education--to be ready to face laboratory emergencies with knowledge, with mastery of technic, with a cool mind and solid judgment, and with a readiness to serve at any hour of the day or night, on Sundays and holidays, regardless of inconvenience and hardship.

This is a part of the medical ethics of the medical technologists in its broad concept, just as it is a part of the medical ethics of the physician.

METHODS OF ANALYSIS

This manual presumes that the laboratory technician has a basic knowledge of general chemistry and its laws. The Handbook of the Hospital Corps, U. S. Navy, 1953, contains an excellent section on the basic laws of chemistry. The special basic chemical principle of each individual determination is outlined as each method is presented in the manual.

There are four general methods in use in chemical analytical laboratories. These are:

1. Gravimetric methods in which the estimation is made by weighing an isolated purified substance, which may have been originally present in the sample or formed by a reaction.

2. Volumetric (titrimetric) methods which are based on the measurement of the volume of a reagent used up during the reaction with a measured amount of sample.

3. Colorimetric methods in which the amount of colored material present or formed by a reaction is measured by light absorption techniques.

4. Gasometric methods in which the amount of a gas present or produced by a reaction is measured either by

- a. Measuring the volume at known conditions of temperature and pressure and water vapor saturation or

- b. Measuring the pressure at known volume, temperature and water vapor saturation.

The apparatus commonly used in these gasometric methods for clinical procedures are respectively (a) the Van Slyke volumetric and (b) the Van Slyke manometric apparatus.

Most of the methods used in laboratories today are colorimetric, one or two are gasometric, a number are volumetric and almost none are gravimetric.

An analytical chemical method to be used in a routine clinical chemistry laboratory must meet the following requirements:

1. It must be accurate enough and precise enough for clinical interpretation.
2. It must require a sample small enough to be obtained without harm to the patient.
3. It must give results in a short enough time that they become clinically useful. A rapid method often is of much greater value to the physician (and to the patient!) even though it may be less precise than a slower one. Every laboratory of necessity often chooses reluctantly between accuracy and speed and accepts the compromises inherent in such a choice.

Volumetric and Colorimetric methods will be discussed further here. Gravimetric methods will not be discussed further as a method of analysis, but since the weighing of materials in the preparation of solutions is a very large and important part of laboratory work, the use of laboratory balances will be further discussed below.

GENERAL CHEMICAL TECHNIQUE

Quantitative analysis differs from qualitative analysis not only in its ultimate objective but in the details of manipulation of the analysis. Quantitative analysis requires great care to achieve the precise measurements necessary to accurate determinations; whereas, in qualitative tests, rather large errors in measurement affect the results only very slightly.

The recommendations and general rules which will be noted here are for the purpose of increasing the accuracy and precision of the individual methods collected herein. The acquiring of skill in chemical technique is much like learning to drive a car or to play the piano. The purpose is to achieve accuracy in a chemical determination and rules are set up to help to do this. Also, like learning these other skills, the time will come when the separate rules are forgotten but the integrated technique remains. But bad habits as well as good ones can be learned, so at the beginning of your instruction, follow to the letter the rules which are outlined for you; always, of course, looking for the reason behind the rule.

CHEMICAL BALANCES

The determination of the weight (mass) of a substance is one of the fundamental physical measurements. In clinical chemistry, a number of different types of

balances are used depending upon (1) the accuracy required in the measurement and (2) the total mass which is to be weighed.

These two factors are somewhat inter-related—the most sensitive balances being designed to handle only very limited loads—the less sensitive being designed to handle greater loads. It is possible, but difficult, to design and construct a balance to be both highly sensitive and able to handle great loads (large capacity).

Types of Balances

It is important to choose the type of balance required for a given job. If we desire to weigh out 10 g. of material with a required accuracy of only 0.5 g. then we should use a trip balance, accurate to about 0.1 g.; the analytical balance should not be used since its accuracy is excessive (0.0001 g.).

1. The analytical balance (Figure 1, p. 5a). This is the most sensitive of the commonly used balances. It is used in the preparation of standard solutions and wherever accuracy to one milligram or less is required. The load capacity is about 100–200 g.

2. The prescription balance (Figure 2, p. 6a). This type of balance has a load capacity of about 100 to 200 g. and is accurate to about 10 mg. (0.01 g.) It is commonly a torsion type balance—depending on the twisting of a wire or a steel tape—and containing no knife edges. Its relatively great sensitivity also requires (as does the analytical balance) that the pans be protected against air currents during weighing.

3. The trip balance (Figure 3, p. 6a). This is a most useful balance. It has a load capacity of about 2000 g. with a sensitivity of about 100 mg. (0.1 g.). It is equipped with separate weights and a sliding beam weight or with an adjustable beam weight plus a slider.

4. Triple-beam balance (Figure 4, p. 6a). This is a very convenient type of balance for general weighing in the laboratory with a capacity up to about 200 g. and a sensitivity of about 10 milligrams. It has only one pan, attached to the short arm of a beam with arms of unequal length. On the long arm, the beam has three graduated weight scales located in a single horizontal plane, each carrying its own attached rider, two of the scales are notched and the third is equipped with a sliding rider.

5. The solution balance (Figure 5, p. 6a). This is of use where large volumes of solutions are being prepared. It is equipped with a sliding beam weight for taring a beaker or other container, as well as separate weights and sliding beam weights. It has a capacity of 20,000 g. (20 kilograms) with a sensitivity of 1000 mg. (1 g.).

There are numerous special types with varying capacities and sensitivities for special uses.

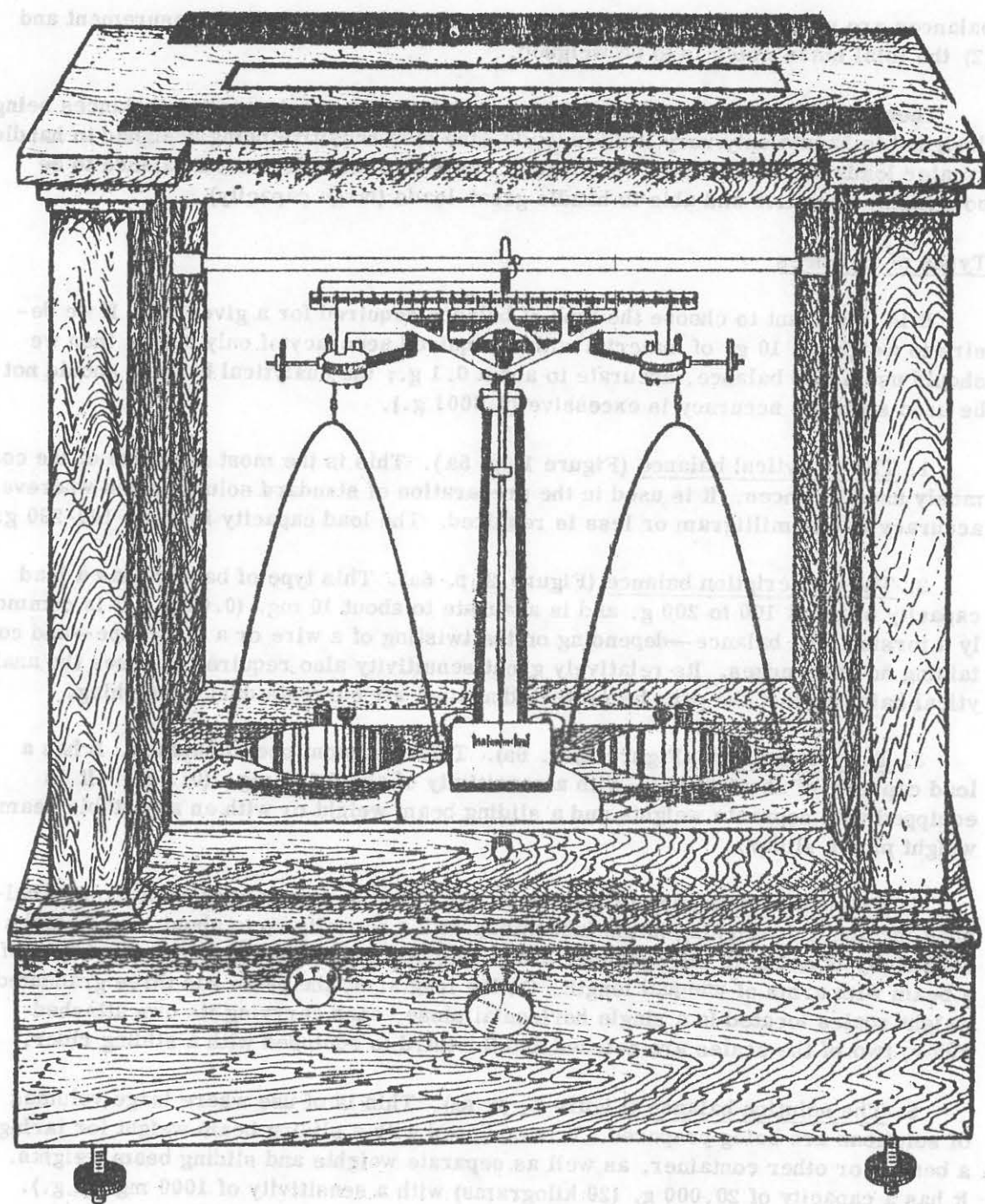


Figure 1. Analytical Balance

Care and Maintenance of Balances - General Principles

1. Balances are, in most instances, constructed with a rigid horizontal beam (or lever) with the two arms of equal length (except in the case of the triple beam (4) and the solution balance (5) above), with two pans suspended at the ends of the beams on knife edge supports. The center of the beam, the fulcrum, is another knife edge lying in the same plane but above the center of gravity of the system.

2. These knife edges and the plane surfaces on which they rest during the weighing operation are the most vulnerable points in a balance. The balance should always be treated with gentle care so as to protect these points of strain.

Analytical and triple beam balances have devices which allow the knife edges and the plane surfaces on which they rest to be separated very slightly whenever objects are placed on or removed from the pans. During times when the balance is not in use, the knife edges should be lifted from the plane bearing surface to prevent wear. The beam should always be lowered slowly and carefully.

3. Most balances must be level for accurate work and are equipped with plumb bobs or spirit-levels of some description which should be checked before any weighing is done.

4. Balances should be located away from exposure to direct heat such as radiators or direct sunlight and should not be exposed to blasts from fans or other sources of rapidly moving air.

All objects must be at the temperature of the balance room before they are weighed. Warm or cold objects produce currents of air in the balance case which interfere with accurate weighing.

5. Substances should never be weighed directly upon the pans of any balance. Instead, special "weighing paper" or tared watch glasses or beakers should be used. The balance should never be exposed to corrosive fumes. If possible a separate room should be used to avoid this. When corrosive substances must be weighed, they should be in a closed weighing bottle.

Special Precautions for the Analytical Balance

1. Never place on or remove from the balance pans any weight or vessel without "arresting" the balance; that is, raising the mechanical supports so that the knife edges no longer touch their bearing surfaces.

2. The beam and pans must be released gently to avoid injury to the knife edges.

3. The balance must never be left with the case open or with the beam unsupported. The rider should be left at the zero position.

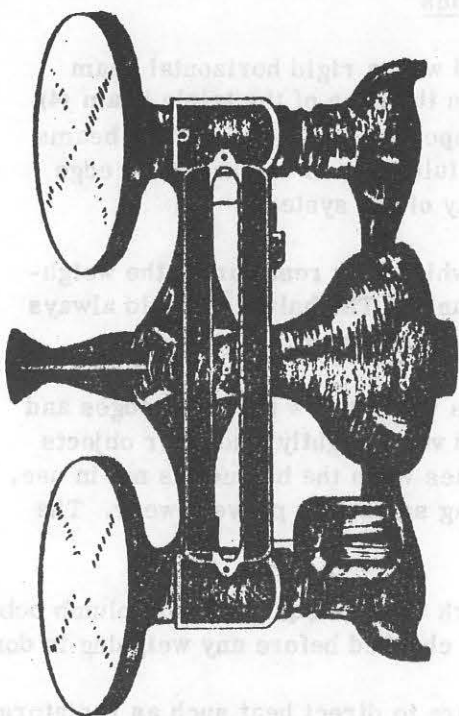


Figure 3. Trip Balance

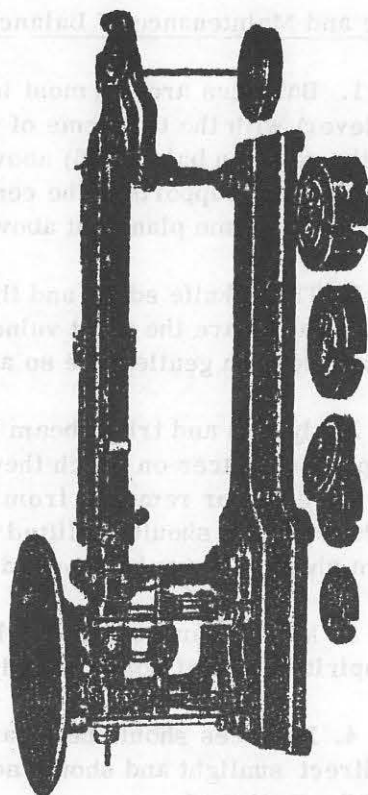


Figure 5. Solution Balance

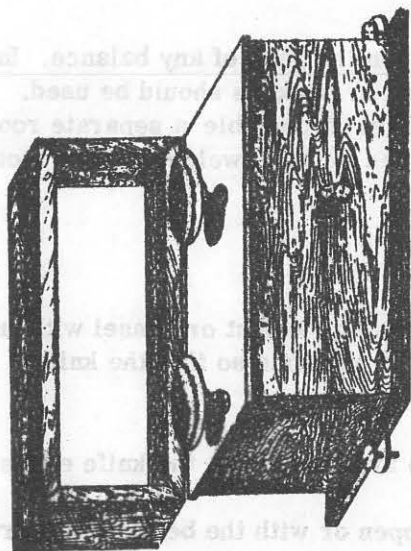


Figure 2. Prescription Balance

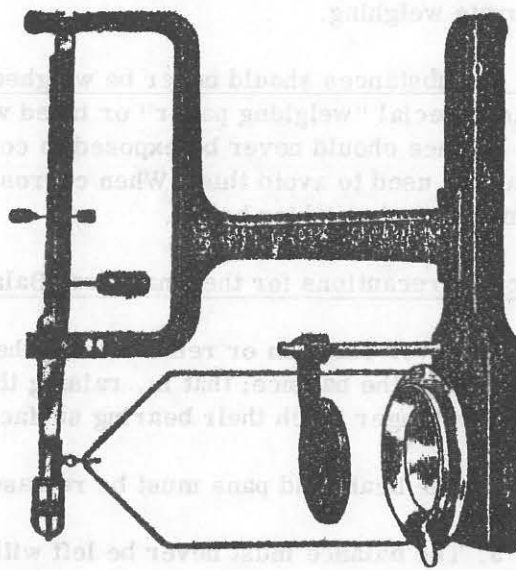


Figure 4. Triple Beam Balance

4. Be careful to avoid spilling materials on the pans or on the floor of the balance case. If this happens, remove at once by dusting carefully with a camel's hair brush.

5. Never set on the pan any vessel having moisture or chemicals on its outside surface. Corrosion may otherwise ensue.

Operation of Balances (other than the analytical balance)

There is usually some device to indicate the neutral or zero point of the balance. The indicator should be centered on the zero point at the beginning of weighing and at the end of the weighing.

Problem: To weigh out 10.5 g. sodium chloride, using the trip balance.

1. Make sure that the balance is level and the adjustments are made so that without any weight on the pans on either side that the indicator is at rest at the zero point.

2. Place a clean dry watch glass on the left pan. Now using the tare beam, or a similar watch glass and glass beads placed on the right pan, bring the balance back to indicate the zero point. At this point, the container is said to be "tared."

3. Now place weights totaling ten grams on the right pan, and adjust the beam scale rider to read 0.5 g. (In some balances there are two beams with a large and a small rider to eliminate the use of loose weights.)

4. Add sodium chloride, by spatula, to the watch glass on the left pan, until the indicator again reads zero, indicating that there is now 10.5 g. of NaCl present.

5. Each balance has its special devices and these should be noted for the most efficient use of each instrument.

Care of the Weights

An instrument of extreme precision is of little use without a set of accurate weights. The weights should be just a shade more accurate than the sensitivity limit of the balance, with which they are to be used. There is little point in using analytical grade weights on a trip balance. Conversely, there is little point in using crude weights with an analytical balance.

Analytical (and indeed all) weights should be treated with great respect and with intelligent care. They should be always kept in a suitable box, each weight in its own proper place. The weights should be removed from the box or from the pan of the balance with a set of forceps, never with the fingers. They should never be left on the balance pans. If a particular weight has lost its polish or lustre do not attempt to restore it. In many sets of weights the following values are found:

50 g., 20 g., 10 g., 10' g., 5 g., 2 g., 2' g., 1 g., 500 mg., 200 mg., 100 mg., 100' mg., 50 mg., 20 mg., 10 mg., 10' mg., and a rider equivalent to a 10 mg. weight. The rider is usually a piece of platinum or aluminum wire looped so that it rests securely on the graduated beam and can be moved conveniently by a special rod provided for the purpose.

Weighing by Swings

Determination of the zero point: The pans and then the beam are carefully released, so as to allow the beam to swing freely, with an excursion of the pointer of about five or six divisions from the midpoint of the scale. Readings are then taken of the extreme points on the scale reached by the pointer in swinging--the "turning points." Two readings are taken on one side of the middle line and one on the other. The first one or two swings are always neglected.

Example: After the pointer returned from its swing to the left, the following "turning points" were recorded:

<u>Left</u>	<u>Right</u>
	6.0 divisions
5.2 divisions	5.6 divisions

The turning point on the right corresponding to the 5.2 on the left is the mean of the two readings on the right, --or 5.8 divisions. Note: that in this case, the turning points are not equidistant from the middle point. (If they had been, the zero point would have been at the middle point of the scale.) If magnitudes to the left are given a negative sign and to the right a positive sign, the true rest point is their algebraic sum divided by two; thus:

$$\frac{-5.2 + 5.8}{2} = +0.3 \text{ divisions}$$

The zero point is thus 0.3 divisions to the right of the middle line. This is the point at which the pointer would stop if it were allowed to come to a stop undisturbed.

This same procedure is used in actual weighing to determine the exact tenth of a milligram (see below).

Weighing by swings: In weighing on the analytical (or other) balance the substance or weights can be added or removed until the original zero point is re-attained. This is a tedious process when weighing to tenths of a milligram. Much time can be saved by estimating tenths of a milligram by noting the amplitude of the swings as above. Weight is added to the right pan and the rider adjusted until the pans are balanced within one milligram. The rest point is then determined as above for the zero point.

The position of the rider is then shifted one milligram and the rest point is once more determined. The weight at the zero point is now determined by calculation as illustrated in the following example:

A coin (a U.S. nickel) was placed on the left pan of a balance with a zero point (previously determined as above) of $\nearrow 0.3$ (the plus notation is used for values to the right of the midpoint of the scale). Weights placed on the right pan amount to 4.99 g. and the rider is located at 8 milligrams to bring the balance within 1 milligram of equilibrium. With the weights totaling 4.998 g. the rest point is now determined to be located at $\nearrow 1.5$ divisions. (1.5 divisions to the right.)

The rider is now shifted to the 9 milligram point on the beam. With the weights now totaling 4.999 the rest point is found to be located at -0.8 divisions (0.8 divisions to the left).

Our data are therefore as follows:

Zero load - zero point is $\nearrow 0.3$

Total weight 4.998, the rest point is $\nearrow 1.5$

Total weight 4.999, the rest point is -0.8

Calculation: One milligram produced a shift from $\nearrow 1.5$ to -0.8 , which is 2.3 divisions; that is, a change of weight of 1 milligram is equivalent to 2.3 divisions. This value is known as the sensitivity, and varies slightly with the load. The zero point does not vary with load.

The additional weight needed to move the pointer from a rest point of $\nearrow 1.5$ to $\nearrow 0.3$ is the weight needed to move the pointer 1.2 scale divisions. $\nearrow 1.5 - (\nearrow 0.3) = 1.2$
Since each milligram moves the pointer 2.3 scale divisions then $\frac{1.2}{2.3} = 0.52$ mg. required to bring the rest point to exactly $\nearrow 0.3$.

Therefore, the nickel weighs $4.998 \nearrow 0.00052 = 4.99852$ g.

The calculation can also be made from the other direction as follows:

Total weight 4.999 rest point -0.8

zero point $\nearrow 0.3$

The weight must be reduced sufficiently to change the rest point from -0.8 to $\nearrow 0.3$. A change of 1.1 scale divisions. $1.1/2.3 = 0.49$ mg.

Therefore the nickel weighs $4.999 - 0.00049 = 4.99851$ g.

Note:

When a precise amount of a chemical must be weighed out, it is advantageous to use a prescription balance to approximate the amount needed. The weighing may then be completed precisely on the analytical balance.

Damped Balances

Some balances are equipped with dampers, either air cylinder or magnetic so that the oscillation may be reduced to one or two swings. This makes it possible to read the rest point directly instead of using the calculations indicated above. Otherwise the procedure is essentially identical.

Chain-O-Matic

Some balances are arranged so that a hanging chain with a variable loop size can be used as a substitute for the rider. These devices speed weighing at very little sacrifice in accuracy and precision. There are many variations in the application of the rider principle and supplementary weights. Each balance should be studied before use to obtain the greatest advantage from these devices.

Calibration of Weights

For the greatest accuracy, the weights used with an analytical balance should be calibrated so that corrections for the inaccuracies can be made. Details of this procedure can be found in books on quantitative analysis or in the following articles:

Richards, T. M., J. Amer. Chem. Soc., 22, 144 (1900)

Blade, E., Indust. and Eng. Chem., Anal. Ed., 11, 499 (1939)

Ainsworth, A. W., Indust. and Eng. Chem., Anal. Ed., 11, 572 (1939)

Craig, A., Indust. and Eng. Chem., Anal. Ed., 11, 581 (1939)

VOLUMETRIC ANALYSIS

Volumetric definitions:

A molar solution of any chemical compound is a solution of such concentration that one liter contains one gram molecular weight of the compound; e.g., the molecular weight of HCl is 36.46 g. A molar solution of HCl contains, therefore, 36.46 g. of HCl per liter; a molar solution of H_2SO_4 (Mol. Wt. 98.08) contains 98.08 g. per liter of solution.

A normal solution of any compound is a solution which contains one gram atomic weight (1.008 g.) of reacting hydrogen per liter, or one which can quantitatively replace or react with an equal volume of such a solution. The fraction of a gram-molecular weight in a liter of normal solution (containing a gram-equivalent weight) depends upon the reaction for which the substance is used. A normal solution of H_2SO_4 , with a molecular weight of 98.08, contains 49.04 g. per liter, because of the presence of two reacting hydrogen atoms per molecule.

In acid-base (alkali) titrations, a normal acid solution contains per liter the amount of acid which has 1 gram atom of hydrogen replaceable by alkali (base) at the pH used as an end point in titration.

A normal alkali solution is one which neutralizes, volume for volume, a normal acid solution. A normal solution of NaOH is molar but a normal solution of $\text{Ba}(\text{OH})_2$ is half molar.

In oxidation-reduction reactions, a normal reducing solution is one of which a liter contains 1 gram atom of oxidizable hydrogen or its equivalent in other reducing substances. Oxalic acid, $\text{H}_2\text{C}_2\text{O}_4$, has two hydrogen atoms, both of which are titratable with alkali, and both of which are oxidizable by permanganate. Thus a normal solution of oxalic acid whether for acidimetry or for oxidation by permanganate, is half molar.

A normal oxidizing solution is one of which a liter will oxidize 1 gram atom of hydrogen, or its equivalent of other reducing substances.

Volumetric apparatus and its use:

In volumetric analysis, cylinders, flasks, pipets and burets and other glassware of various types are used. These consist of containers very accurately graduated either "to contain" or "to deliver," very accurately, a certain volume of solution, usually aqueous. Measuring cylinders are graduated in milliliters and are used for measurements where a high degree of accuracy is not required, and when properly used, are suitable for many measurements. For good work, one should always attempt to obtain the greatest accuracy possible with the instrument at hand. All apparatus for accurate work should be calibrated by the chemist himself.

In order to obtain satisfactory results in the measurements of liquids in such vessels, it is necessary to learn to read the meniscus properly. With transparent solutions the bottom of the meniscus is read; with deeply colored and non-transparent solutions, such as strong permanganate solutions and blood, the reading is made at the top of the column of fluid. In making a reading of the volume in a buret or flask, it will be found that as the eye is raised or lowered the apparent position of the liquid meniscus alters. To avoid such errors (parallax) the reading must be made with the eye at the level of the top of the column of fluid. Most vessels have graduations which completely encircle the tube or neck to help eliminate errors due to parallax.

Cleaning of apparatus:

Detergents: There are many non-ionized, synthetic detergent products available which do an excellent job of cleaning glassware. Their efficiency is increased many-fold if used hot. There is a tendency to use too concentrated a solution; about a 1% solution of most products works very well.

"Cleaning solution": One of the most efficient solutions used for the cleaning of volumetric glassware is composed of various concentrations of sulfuric acid saturated with a dichromate salt but it must be handled with the greatest of care since it is an oxidizing acid solution and attacks and destroys skin, clothes, desks, floors and books, etc. It should be chosen for use only after detergents have been tried and found wanting.

- | | |
|--|-----------|
| I. $K_2Cr_2O_7$ | 100 grams |
| Concentrated sulfuric acid (tech.) ... | 250 ml. |
| Water | 750 ml. |

Note: The concentrated sulfuric acid should be poured into water.

- | | |
|-------------------------------------|-----------|
| II. $Na_2Cr_2O_7 \cdot 2H_2O$ | 200 grams |
| Water | 100 ml. |

Dissolve, cool. Pour into this solution with continual stirring 1500 ml. of concentrated H_2SO_4 (tech. grade). This cleaning mixture is strong and more dangerous and should be used only with dry glassware since salt precipitates out if it is diluted with water.

The purpose of special cleaning procedures for volumetric glassware is to maintain the apparatus free from grease which when present causes the surface of the glass to be "non-wetting" and droplets of aqueous solutions then will collect on the surface rather than spreading in the thin film which is essential to accurate results. If the failure to "wet" is due not to grease but to "silicones" the problem of removal becomes more difficult. The use of alcoholic KOH overnight may be helpful.

After use of any of the above, the cleaned apparatus should be rinsed many times with tap water and finally several times with small amounts of distilled water. During the rinsing, the fingers should be kept off the cleaned inside surfaces which are of volumetric importance--fingers are greasy and will easily contaminate the cleaned glassware so as to require re-cleaning.

Drying of apparatus:

Sometimes it is desirable to dry the apparatus quickly. This can be done (a) by rinsing with alcohol, followed by ether, or (b) by rinsing with acetone or (c) by heat. In any case, use of a current of air hastens evaporation. Note, however, that these procedures are desirable only when the apparatus must be dry. Usually it is better just to rinse the apparatus with some of the solution to be measured, if sufficient solution is available. Rinse several times with small amounts of solution, allowing good drainage between rinses. Three rinsings with 5 ml. are better than two with 10 ml., or one with 30 ml. When air is used for drying it must be clean. A drying oven is very convenient for large numbers of apparatus but is somewhat slow. It must be set at a temperature above the boiling point of water for useful application. It has recently been shown that heating pyrex volumetric apparatus (volumetric flasks and pipets) to very high temperatures (300°C.) does not impair their accuracy to a degree noticeable in the clinical chemistry laboratory. J. Chem. Ed. 33, 609 (1956).

Common volumetric glassware: (See Fig. 6 - 13, pp. 13a, 14a)

1. Graduated cylinders are relatively inaccurate measuring devices and are used for situations in which accuracy of a high order is not required. They can, however, be used very conveniently for the measuring of 24-hour urine volumes of 500 to 2000 ml. where the accuracy afforded is quite sufficient.

2. Volumetric flasks are designed to allow accuracy to one part in a thousand (0.1%). They are flat-bottomed, pear-shaped vessels with long narrow necks. The U. S. Bureau of Standards has issued bulletins outlining the requirements which they recommend for the highest accuracy and most manufacturers comply with these recommendations. The long narrow necks make it possible to adjust the final total volume with ease and precision. The neck is encircled with a thin line indicating the capacity of the vessel to that mark, at some definite temperature usually 20°C. In volumetric flasks the mark usually indicates the "contained" volume. In some older German flasks two marks have been made, indicating a "to deliver" and a "to contain" calibration. Most flasks of recent origin have only a "to contain" calibration.

Volumetric flasks are used to prepare solutions of substances so as to have a certain known concentration. The required (calculated) amount of the substance is weighed or measured out and transferred to the flask, water is added to about 1/2 of the volume of the volumetric flask. The substance is completely dissolved, the volume is made up to the mark, avoiding parallax and completely mixed. It is good practice to invert, and mix at least ten times after bringing to volume.

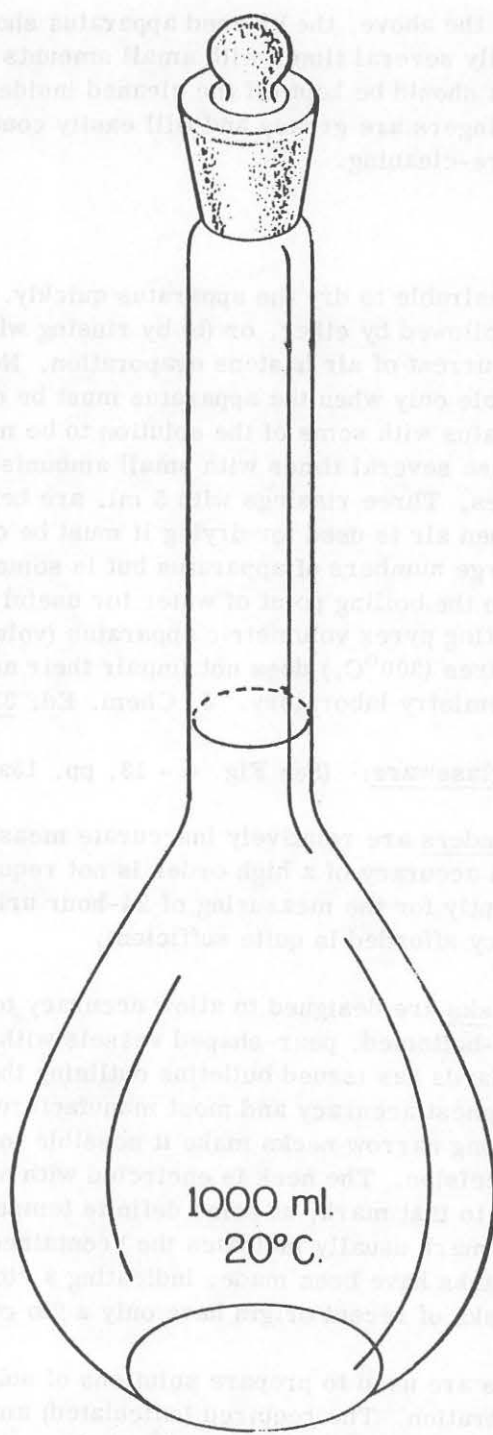


Figure 6. Volumetric Flask

Mild heat may be used in some cases to speed the dissolving but the final adjustment to the mark should be made only after cooling to the calibration temperature.

In no case should the prepared solution be stored in the volumetric flask since they are subject to breakage and etching. Volumetric flasks are not inexpensive.

3. Pipets are cylinders drawn out at one end to form a delivery tip and at the other to form a mouth-piece (since the pipet is usually filled by mouth suction). They are graduated at a single mark on the upper limb of the pipet to fix the volume of fluid which will be delivered under certain specified conditions. Some pipets have many subdivisions and are employed for measuring out variable amounts of fluid. These are of lower accuracy.

a. Transfer pipets (Fig. 7, p. 14a) are calibrated to deliver a certain volume of fluid. The accuracy possible is of the same order as volumetric flasks (0.1%). In order to attain this accuracy the orifice must be of such a size that the outflow of the fluid is not too rapid for otherwise slight differences in drainage time give large variations in delivery volume. The Bureau of Standards has also set up requirements for this type of pipet.

b. Measuring pipets (Fig. 8, p. 14a) or "Mohr" pipets consist of a cylinder of uniform bore calibrated with multiple graduations; they have a lower order of accuracy and are made to deliver "between marks." That is, they are not allowed to drain to the tip but the measurement is made between two marks on the pipet.

c. Serological pipets (Fig. 9, p. 14a) are not as accurate as transfer pipets and are used when their margin of error will not affect the results of the determination. Both the measuring pipet and the serological pipet are graduated assuming that the internal bore is of uniform diameter and this is not always true. Titrations should never be made using measuring or serological pipets. They are usually "blow-out" pipets and are designed to deliver as noted below under Ostwald-Folin type.

d. Ostwald-Folin pipet (Fig. 10, p. 14a) is a pipet especially designed with a large oval bulb and a short delivery tip so as to minimize the effects of viscous substances in the measurement of the fluid volume. It is used in the measurement of blood, serum or plasma. It is calibrated to deliver "by blow-out." That is, the pipet is allowed to drain to the tip and then the small amount of fluid that remains in the tip after drainage is expelled by closing the upper end with the forefinger and warming the bulb by gripping it with the palm of the other hand. The warmed air expands and forces the last drop out of the tip which is held against the side of the receiving vessel.

e. Capillary pipets (Fig. 11, p. 14a) are usually calibrated "to contain" and should be rinsed out thoroughly 6-8 times with the diluting fluid used in the particular determination being carried out.

Transfer
pipet



Figure 7

Measuring
pipet



Figure 8

Serological
pipet



Figure 9

Ostwald-Folin
pipet



Figure 10

Capillary
pipet



Figure 11

Folin-Wu
pipet



Figure 12

Buret

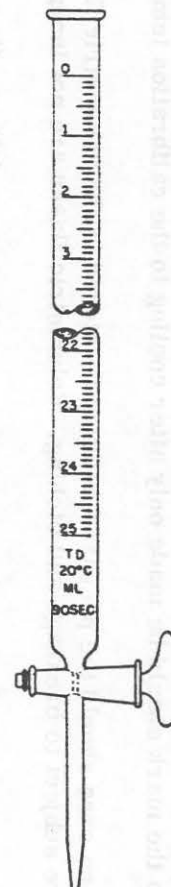


Figure 13

f. "Folin-Wu" pipets (Fig. 12, p. 14a) are a special type of measuring pipet designed to measure the fluids involved in the preparation of the tungstic acid protein free filtrate (p. 76). It is designed to drain to the tip and is not a "blow-out" type.

4. Burets (Fig. 13, p. 14a) are graduated tubes with a uniform bore which are used for measuring the volume of solutions in titrations. They are available in many sizes, of which perhaps the 50 ml. capacity is the most commonly used. Micro-burets are those with a total volume of 5 or 10 ml. down to those of 0.1 ml. total volume.

They should be thoroughly clean before use so that a uniform film of liquid remains on the walls when the liquid is allowed to flow out. Care should be exercised in reading a buret to place the eye on a level with the lower part of the meniscus of the solution, so that parallax will not introduce serious errors. In measuring with the buret always allow one minute to elapse before taking a reading. This allows time for drainage from the walls of the buret.

Outlet valves to control the flow of liquid from burets are in general of two types: (a) a ground-glass stopcock which gives excellent control and can be used with most solutions although strong alkali solutions tend to etch and "freeze" the stopcock if they are allowed to remain for long periods; (b) a rubber tubing connection between a glass tip and the end of the buret is the main second type. The control is effected either by a pinch-clamp or by a glass bead which obstructs the lumen of the rubber tubing and is controlled by a pinching of the rubber to one side of the glass bead, to produce a slight crease through which liquid can flow.

Glass stopcocks require some care in lubrication in order that they may give good service and still avoid undue plugging of the bores. A minimum of lubricant should be used. The stopcock with its adjoining connections must be absolutely clean before it is lubricated. Commercial pipe cleaners are useful in cleaning connections and bores. Old grease may be removed by scrubbing with a little ether. Lubricant which enters the bore of the stopcock must be removed. Such entrance may be minimized by applying only a minimal amount of grease in a ring about both ends of the stopcock and working this in by turning the core back and forth in its casing without complete rotation. A well lubricated stopcock should appear transparent throughout and should turn with perfect ease. Excessive pressure should not be exerted on the cock during lubrication or in the subsequent use of the apparatus. Such pressure tends to drive out the lubricant and to wear out the cock. If bits of lint or other foreign materials are left between the core and casing, they will etch the cock during lubrication and cause channels with resultant leakage.

Micro-burets are of many different types. The syringe screw burets are very convenient and many use a micrometer caliper with a plunger to force and to measure the delivery of the fluid. The micrometer scale is then used in reading.

Notes:

"Blow-out" type pipets commonly are identified by an etched or sandblasted ring near the upper end.

One should always inspect the markings on a buret or pipet carefully to determine the capacity, the type, and especially the calibration intervals. Both burets and pipets are calibrated to deliver water, and will not deliver with equal accuracy fluids that differ greatly from water in viscosity, cohesion or surface tension. When water is run out of a buret or pipet, a film of liquid remains adherent to the walls. The more rapid the rate at which the vessel is emptied, and the greater the viscosity of the fluid, the thicker this film will be. In order to deliver amounts constant even to within one part per hundred, it is necessary that the surface of the liquid descend in the vessel at a sufficiently slow and steady rate. In this way the residual film will be thin and constant.

Precautions in the Use of Pipets:

1. Never insert a pipet into the stock reagent bottle. If the pipet is dirty or some untoward incident occurs, the entire stock of reagent may be ruined. Instead, pour some solution out into another container (a beaker or test tube) and pipet from this. Discard the excess reagent—never return the excess to the stock bottle.

2. The pipet must be clean and show no droplets on the wall after draining.

3. Before use the pipet must be dry or rinsed three times with the solution it is to measure.

4. Do not pipet poisonous or corrosive reagents without safety devices such as a long rubber tube between mouth and pipet.

5. Fill the pipet to above the mark with the solution to be transferred, using gentle suction with the mouth and then close the upper end of the tube with the dry index finger. The tip of the pipet is then wiped off to remove any adhering droplets, and the solution is then allowed to flow out slowly with the pipet held in the vertical position, until the meniscus coincides with the mark, and the tip is then "touched off" to the side of the vessel. The pipet is carefully moved to the delivery vessel and the fluid allowed to deliver freely with the tip in contact with the inclined wall of the vessel. After free out-flow has ceased, keep the tip in contact with the wall for about fifteen seconds.

6. If the pipet is the blowout type expel the fluid in the tip as outlined above under the Ostwald-Folin pipet. If it is the "to contain" type it must be rinsed out as outlined under that type.

Volumetric analysis - general principles:

In volumetric analysis, one solution (generally in a buret) is added to a carefully measured volume of another reagent, in a beaker or flask. The addition from the buret is continued until the reaction is seen to be complete. This is known as the "stoichiometric end-point" or merely as the "end-point" of the titration. It is usually indicated to the analyst by a rapid change of some sort occurring in the solution such as a change of color, turbidity, etc. Very often, the change is due to a change in an auxiliary reagent known as an "indicator" which has been added for the purpose. In other cases the end point may be indicated by the appearance of an excess of the titrating substance in the solution being titrated. The titrant itself is thus serving as its own indicator.

In acid-base titrations the indicators are weak acids or bases which have a different color in the form of their salt. In oxidation-reduction indicators, the oxidized form of the indicator has a different color from that of the reduced form. The choice of an indicator will depend upon the particular reaction being carried out and it should indicate the point of chemical equivalence of the two reactants - that is the "stoichiometric end-point."

For acid-base reactions it may be said that, in general, the indicator should show its color change at an acidity (or alkalinity) corresponding to that of a solution of the pure salt formed in the titration. For example: the pH or $-\log(H^+)$, (which is a measure of the acidity of the solution) (see page 40) of a 0.05 N solution of sodium acetate which is the product of the reaction of 0.1 N NaOH and 0.1 N CH_3COOH (acetic acid) is about 8.8, slightly on the alkaline side of acid-base neutrality (pH 7). Therefore, to attain stoichiometric or chemical equivalence at the end-point, we should choose an indicator such as phenolphthalein which changes color close to 8.8 (instead of one changing close to pH 7).

In the case of the titration of 0.1 N HCl with 0.1 N NH_4OH , the solution at the stoichiometric end-point will be 0.05 N NH_4Cl with a pH of about 4.6; the indicator of choice here is one that changes color on the acid side of acid-base neutrality close to 4.6 so as to achieve stoichiometric equivalence at the end-point. Methyl orange or methyl red are such indicators. The choice of an indicator is not always a simple matter. It is best to use the indicator recommended in the procedure used, if at all possible.

Volumetric standards, like volumetric measuring apparatus, can be accurate to one part in 1000. For convenience in computation, concentrations of solutions are expressed in terms of normality, i.e., 2 N, 0.5 N, or 0.01 N. The capital N, underlined, is the usual abbreviation for normal. For further definitions see p. 11.

A given number of ml. of any solution is equivalent to the same number of ml. of any other solution of the same normality if it is used for the same type of reaction. Thus 1 ml. of 0.5 N HCl is equivalent to 1 ml. of 0.5 N KOH and equivalent to 1 ml. 0.5 N H_2SO_4 because each is equivalent to the same amount of hydrogen. If one has a

solution of either acid or alkali whose normality is known, the strength of other acids and alkalies can be determined with ease.

Since the molecular weight of HCl is 36.46 and of NaOH is 40.00, it follows that 36.46 g. of HCl contain the same number of molecules as 40.00 g. of NaOH. If 36.46 g. HCl and 40.00 g. of NaOH are each dissolved in water and diluted to exactly one liter, each liter will contain the same number of dissolved molecules, and each ml. will contain the same number of molecules. The two solutions are equivalent to each other and are each 1 (one) normal in concentration.

The HCl is 1 (one) normal because it contains 1 g. (1.0080 g.) of replaceable hydrogen in one liter of solution, and the NaOH is 1 (one) normal because it contains 17 g. (precisely 17.0080 g.) of hydroxyl ion (OH^-) which is equivalent to 1 g. of hydrogen. The molecular weight of H_2SO_4 is 98. A solution containing 98 g. of H_2SO_4 per liter contains the same number of molecules per unit volume as the HCl solution of 36.46 g. HCl per liter. But each molecule of H_2SO_4 contains 2 atoms of replaceable hydrogen. The solutions are not equivalent since the H_2SO_4 contains 2 g. of replaceable hydrogen per liter; it is twice as strong as the HCl or $2 \times \text{N}$. To make a normal solution of H_2SO_4 , take $98/2 = 49$ g. H_2SO_4 per liter of solution. Thus a normal solution contains one equivalent weight of the substance in a liter of solution. Then one milliliter of a normal solution will contain one milliequivalent weight of the substance, or the same number of milligrams of the substance as one liter contains grams of it. Hence 1 ml. of a 1 N solution of HCl contains 36.46 mg. of HCl; 1 ml. of 1 N NaOH contains 40 mg. of NaOH; 1 ml. of 1 N H_2SO_4 contains 49 mg. of H_2SO_4 . The number of milliequivalents (abbreviated meq. or mEq.) of a substance present in a given volume of a 1 N solution is equal to the number of ml. If the solution is 0.5 N, there is in each ml. 0.5 meq. and in 10 ml. there would be $10 \times 0.5 = 5$ meq.

It is always true that the product of the number of ml. times the number of milliequivalents in each ml. (the normality) gives the total number of meq. present. Since dilution does not change the amount of solute present, the volume of the original solution (V_1) times its normality (N_1) equals the volume of the diluted solution (V_2) times the new normality (N_2), or

$$N_1 V_1 = N_2 V_2 = \text{meq present}$$

If three of these quantities are known, obviously the fourth can be calculated.

If it is established by titration that a given volume of an unknown solution (V_1), requires a determined volume (V_2) of a solution of known normality (N_2) to reach an equivalence point (stoichiometric end-point), the same equation can be used to calculate the normality of the unknown solution, N_1 .

GENERAL INSTRUCTIONS FOR THE PREPARATION OF SOLUTIONS

Certain standard solutions can be directly and accurately prepared by weighing out a calculated amount and dissolving up to a predetermined volume. These are known as primary standards. Others are standardized directly or indirectly against those which have been prepared by weight directly. All solutions are so prepared that a certain quantity of solute is contained in a known volume. In general, volumetric standards, like volumetric measuring apparatus, should be accurate to one part in a thousand. Normal and fractional normal solutions are not always stable and must be rechecked if they have been standing some time. These instabilities will be noted under each solution as it is mentioned.

In many instances, solutions are required, whose concentration need be known less accurately or in which small variations are not important to the results of the analysis. In these the concentration may be expressed less precisely, commonly as a percentage. By this (for our use) is meant grams of substance per 100 ml. of solution and is sometimes expressed as "W/V" or "weight per volume." Where fluids are dissolved in fluids the short-hand form "V/V" may be used.

The attempt to be concise in the description should not lead to ambiguity. Such expressions as "dilute one to three with water" are not precise and lead to mistakes. Also when the article of commerce is not a pure substance but a solution in water such as concentrated HCl (about 37% HCl by weight) a description of a solution as a 10% solution of HCl does not allow the reader to choose with any degree of certainty between 3.7% by weight of HCl and 10% by weight HCl.

Accurate solution preparation is essential to good analytical work. However, the degree of accuracy required varies from one solution to another. Primary standards as discussed below should be prepared with maximum volumetric accuracy, i.e., to about 0.1%. Other solutions, because of the less critical part they play in the reaction may need to be accurate only to 1% or so. In the description of solutions in each method an indication of the degree of accuracy required is shown by the number of significant figures included.

Thus, the weight of a primary standard required will be stated to tenths of a milligram (as 4.6827 g.) while the weight of ammonium oxalate required in a saturated solution will be stated to only two significant figures (as 15 g.).

Technique of solution preparation:

In general, the technique of the preparation of solutions is to measure as accurately

as necessary by weighing, pipetting or by using a graduated cylinder, etc., the required amount of solid or liquid and then by adding water or other fluids to bring to a specified volume. Only in the case of the preparation of primary standards or of dilution of accurately standardized solutions or in the dilutions of unknown solutions, is it necessary to use volumetric flasks. Their use should be avoided where their accuracy is not needed because they are both fragile and costly. Solutions prepared in volumetric flasks should be transferred immediately to other containers for storage.

When diluting acids and bases or in any case where heat is generated by dilution, ALWAYS POUR ACID (OR ALKALI) INTO WATER, to avoid local boiling and spattering of the solution. All solutions should be adequately mixed to homogeneity. A good rule in using volumetric flasks is to invert, shake well and then bring to an upright position again, allowing time for the air bubble to travel to the top each time. This should be repeated at least ten times after all solid material has disappeared from the solution.

PRIMARY STANDARDS

All volumetric and indeed all quantitative determinations depend on the availability of pure compounds and the ability to prepare accurate stable solutions. The pure substances which are best for this purpose are called "primary standards." A primary standard should have the following characteristics.

1. Stable and definite composition.
2. Stable to drying without decomposition.
3. Large equivalent weight (to minimize the weighing error).
4. Analyzable with accuracy.
5. The reaction in which it is involved as a standard should be
 - a. Single
 - b. Well defined
 - c. Rapid
 - d. Complete

Some of the presently available primary standards, their preparation, and some of their uses are listed below.

Primary Standards:

Acids:

1. Constant Boiling HCl M.W. 36.46

Ref: Hylett, G.A., and W.D. Bonner. J. Am. Chem. Soc., 31, 390 (1909).

Standard hydrochloric acid is easily prepared by first preparing diluted HCl by adding about 850 ml. of distilled water to about 1000 ml. of concentrated HCl (analytical

grade). This mixture is then slowly distilled in an all-glass still at the rate of 3-4 ml. per minute. After about 3/4 of the mixture has been distilled, the rest of the distillate is collected until not less than 50-60 ml. remain in the flask. This distilled fraction is constant in composition (that is, it does not change with time) and is not noticeably hygroscopic or volatile. The exact composition of the distilled fraction depends only upon the barometric pressure at the time of distillation, according to the following table.

Barometric Pressure	% HCl by weight	Weight of HCl solution needed for one mole of HCl (grams)
770	20.197	180.407
760	20.221	180.193
750	20.245	179.979
740	20.269	179.766
730	20.293	179.555

By the use of a small pipet, it is a simple matter to weigh out the required amount of acid to less than 10 mg. and this is sufficiently precise to provide acid more accurate than that attained by most other procedures. The weighing of this acid in an analytical balance is permissible since it is non-volatile, but care should be taken to avoid spilling of even small droplets since the liquid is, of course, corrosive.

2. Potassium Acid Phthalate - $\text{KHC}_8\text{H}_4\text{O}_4$ M.W. 204.22

This can be obtained in very pure form (99.95%) and is highly recommended as a primary standard. This salt crystallizes without water of hydration, it is not hygroscopic, and may be dried at 110-115°C. without any danger of decomposition. It is obtained from many different manufacturers and is available from the National Bureau of Standards for a reasonable fee. The latter supplies two types, one especially for titrimetric standardization, and the other for a pH standard (see below).

0.1 N $\text{KHC}_8\text{H}_4\text{O}_4$ -- Weigh out 10.207 g. of dry potassium acid phthalate on an analytical balance and dissolve the crystals in about 200 ml. of distilled water. Transfer quantitatively to a 500 ml. glass-stoppered volumetric flask, add distilled water to the mark and mix. This solution should be kept in a glass stoppered Pyrex bottle and stored in the refrigerator when not being used. It may be used to standardize alkali (except ammonia and other weak bases) using phenolphthalein as an indicator. This solution should be reasonably constant for many months if handled with ordinary care.

3. Potassium bi-iodate - $\text{KH}(\text{IO}_3)_2$ M.W. 389.94

This salt is a strong acid and can be used with any indicator with a color change between pH 4 and 10. It is anhydrous, non-hygroscopic and thus can easily be kept without change on the shelf.

Standardization of Alkali:

Procedure:

1. Using standard acid solutions: Into a 250 ml. Erlenmeyer flask, pipet accurately 25.00 ml. of the standard HCl solution. Add two to four drops of 1% alcoholic phenolphthalein indicator solution and add the alkali (e.g., NaOH solution) from a buret to a permanent light pink color using care at the end-point. The same technique can be used for standardization by potassium acid phthalate but weak bases (e.g., ammonia) should not be used.

2. Using solid standards: 0.8 to 0.9 g. samples of $\text{KHC}_8\text{H}_4\text{O}_4$ or 1.5 to 1.7 g. samples of $\text{KH}(\text{IO}_3)_2$ are weighed out in beakers or flasks very accurately using an analytical balance. The salt is dissolved in about 25 ml. of water and titrated with the base to be standardized using phenolphthalein as the indicator. The normality of the base is calculated as illustrated in VOLUMETRIC CALCULATIONS page 32, using the molecular weights given above and assuming the salts act as acids with one replaceable hydrogen per molecule.

Bases:

1. Sodium tetra-borate (borax) $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ M.W. 381.43

Sodium tetraborate decahydrate is obtained in a pure condition by two recrystallizations of a good product from water, followed by drying to constant weight in a desiccator over a solution saturated with respect to both sucrose and sodium chloride. To expedite drying, washing with 95% alcohol and with ether, as recommended by Hurley, F.H., Anal. Chem. 8, 220 (1936) and *ibid.*, 9, 237 (1937) may be resorted to. In tightly closed containers the decahydrate seems to have considerable stability up to at least 12 months.

2. Tris (hydroxymethyl) amino methane $\text{H}_2\text{N} \cdot \text{C}(\text{CH}_2\text{OH})_3$ M.W. 121.14

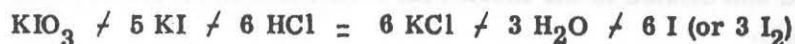
This organic base can be regarded as a monovalent base and has a number of important advantages.

- (1) Relatively high equivalent weight
- (2) Low moisture absorption - about that of potassium acid phthalate - requires no further drying
- (3) CO_2 absorption is absent
- (4) Long shelf-life stability
- (5) Reacts stoichiometrically

Oxidation-reduction:

1. Potassium iodate (KIO_3) M.W. 214.02

Potassium iodate, can be secured in an extremely pure form and used as a primary standard. The salt should be dried to constant weight in a desiccator or in a drying oven ($105^\circ\text{C}.$). Each molecule of this salt, when reacting with an excess of potassium iodide in the presence of acid, liberates 6 atoms of iodine.



Therefore a liter of 0.1 N iodate contains 1/60 of a gram-molecular weight. 0.1N KIO_3 is prepared by weighing out 3.5670 g. of KIO_3 on the analytical balance. Dissolve the salt in 200 ml. of distilled water, transfer quantitatively to a one liter volumetric flask and dilute with distilled water to the mark. This solution should be stored in a glass-stoppered bottle in a cool place. It should keep indefinitely.

2. Potassium bi-iodate $\text{KH}(\text{IO}_3)_2$ M.W. 389.94

This salt, which can be regarded as an equi-molecular mixture of KIO_3 and HIO_3 can be obtained pure and is non-hygroscopic even in the anhydrous state. The reaction is similar to that given above for iodate but one molecule of the bi-iodate liberates 12 atoms of iodine so that the preparation of a liter of 0.1 N $\text{KH}(\text{IO}_3)_2$ (for this reaction) requires only 3.2495 g. Note that above under acid primary standards to prepare a 0.1 N solution to be used in acidimetry would require 38.9940 g. per liter.

3. Sodium oxalate (Sorenson salt) ($\text{Na}_2\text{C}_2\text{O}_4$) M.W. 134.01

This salt can be obtained in high purity. It is recommended over oxalic acid for the standardization of permanganate because when prepared as indicated it forms a very stable solution. When exposed to light a slow decomposition may take place yielding carbon dioxide, carbon monoxide and water.

0.1 N sodium oxalate is prepared as follows: Dry about 10 g. of the pure salt in a drying oven at $150^\circ\text{C}.$ for 3 to 4 hours. Cool in a desiccator. Weigh out 6.7010 g. (M.W./20) on an analytical balance, dissolve in distilled water and transfer quantitatively to a liter volumetric flask. Add 30 ml. of concentrated sulfuric acid (analytical reagent). Cool, dilute to the mark with distilled water and mix thoroughly. It should keep at least a year. The sulfuric acid is essential to long-term stability.

Argentimetric and Mercurimetric.

1. Silver Nitrate (AgNO_3) M.W. 169.89

Very pure preparations of silver nitrate can be obtained. Working in subdued light, the crystals should be pulverized and then dried for 3 to 4 hours at $150^\circ\text{C}.$ They should be stored in a desiccator protected from light.

To prepare a 0.1 N solution of AgNO_3 proceed as follows: Weigh out exactly 8.4945 g. of the dried salt, transfer to a 500 ml. volumetric flask and dilute to the mark with distilled water. Mix. Keep in a brown bottle in the dark.

2. Sodium Chloride NaCl M.W. 58.45

It is convenient to have available a standard chloride solution to check the silver nitrate (or mercuric nitrate) solutions after a lapse of time. For our purposes the analytical reagent grade sodium chloride is sufficiently pure. 0.1 N NaCl can be prepared as follows: 2.9225 g. of the salt which has been dried at 150°C. for 12-14 hours is weighed out on an analytical balance, transferred quantitatively to a 500 ml. volumetric flask and dissolved and diluted to the mark with distilled water. Mix well and store protected from the light.

3. Potassium thiocyanate KSCN M.W. 97.18

Prepare the anhydrous salt by recrystallizing from water one to three times, dry over P_2O_5 in a desiccator for three days, heat at 150°C. for one hour, melt at 200°C. for 10-20 minutes, cool, grind to a powder and store over P_2O_5 , for a few days. Thereafter, it is stable in atmospheres less than 45% relative humidity. For a 0.1 N solution dilute 4.8590 g. to 500 ml. in a volumetric flask.

pH Standards: for the glass electrode

It is always best to use a pH standard which is close to that of the unknown. It is also best to use two different pH standards so as to check the response slope of the instrument, and thus to insure the correct operation of the instrument before readings are made. See below under pH meter, glass electrode p. 46.

Acid Standards:

1. Potassium acid phthalate 0.05 M pH = 4.01 \pm 0.01 at 25°C.

Weigh out on an analytical balance 10.211 g. of pure dry salt, dissolve up to 1 liter in a volumetric flask. Alternatively, it may be prepared from a stock 0.1 N solution (see acid primary standards). This dilute solution is good for a few days and then should be discarded.

2. Potassium acid tartrate 0.034 M pH = 3.56 \pm 0.01 at 25°C.

An aqueous solution saturated at between 22 - 28°C. An error of 10% in concentration produces the given pH with an error of about 0.01 units.

3. Potassium tetraoxalate 0.05 M $KH_3(C_2O_4)_2 \cdot 2H_2O$ pH = 1.68 \pm 0.01 at 25°C.

A solution of this salt recrystallized from water is pure enough for the preparation of the standard. Dissolve 12.7095 g. and dilute to 1 liter with water.

4. Hydrochloric acid 0.1 N pH = 1.10 \pm 0.02 at 25°C.

Prepare exactly 0.1 N HCl as indicated under secondary standards p. 28.

Alkaline Standards

Standards for pH values higher than 7 are complicated by the absorption of CO₂ from the atmosphere and the presence of CO₂ in the water used in the preparation. Freshly boiled water should be used and the standards should be protected from the atmosphere as much as possible.

1. Sodium tetraborate decahydrate 0.01 N Na₂B₄O₇ · 10 H₂O

pH = 9.18 \pm 0.01 at 25°C.

Dissolve 3.8143 g. of the pure salt in 1 liter freshly boiled, cooled distilled water. The presence of slight amounts of the pentahydrate will not affect the final pH to any significant degree. This is not of course true when the acidimetric standard is being prepared.

2. Sodium hydroxide 0.1 N NaOH pH = 12.88 \pm 0.03 at 25°C.

Prepare as described under stock reagents below, (see p. 28) and standardize and adjust to exactly 0.1 N.

4. Hydrochloric acid 0.1 N pH = 1.10 ± 0.02 at 25°C.

Prepare exactly 0.1 N HCl as indicated under secondary standards p. 28.

Water Standards

Standards for all values higher than 7 are compensated by the absorption of CO₂ from the atmosphere and the presence of CO₂ in the water used in the preparation. Freshly boiled water should be used and the standards should be protected from the atmosphere as much as possible.

1. Sodium tetraborate decahydrate 0.01 N $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$

pH = 8.10 ± 0.02 at 25°C.

Dissolve 3.5105 g. of the pure salt in 1 liter freshly boiled, cooled distilled water. The presence of slight amounts of the pentahydrate will not affect the final pH to any significant degree. This is not of course true when the sedimentary standard is being prepared.

2. Sodium hydroxide 0.1 N NaOH pH = 12.95 ± 0.02 at 25°C.

Prepare as described under stock reagents below (see p. 28) and standardize and adjust to exactly 0.1 N.

SECONDARY STANDARDS

Certain substances, although relatively stable once a solution has been prepared, do not have the characteristics required of a primary standard, (see p. 20). They are prepared in approximately the correct concentration, and then their exact concentration is determined by titration against the appropriate primary standard.

Although the method of standardization of working secondary standards is usually given in detail under each particular method, a recommended procedure will be outlined here for some, since these solutions are of general application in volumetric work.

Stock solutions:

In the following list are included the usually available acids and alkalis giving the data required for the preparation of more dilute solutions.

Name %(W/W) Formula	Specific gravity	Molecular Weight	<u>N</u> of conc. reagent	Ml. conc. reagent needed for 1 liter 1 <u>N</u> solution
Acetic acid 99.5% CH_3COOH	1.049	60.05	17.4	57
Hydrochloric acid 36.0% HCl	1.19	36.47	11.6	86
Nitric acid 69.5% HNO_3	1.42	63.02	15.4	65
Phosphoric acid 85.0% H_3PO_4	1.71	98.00	14.6 <u>M</u>	68 (1 <u>M</u>)
Sulfuric acid 96.0% H_2SO_4	1.84	98.08	17.8 <u>M</u>	28
Sodium hydroxide 48% NaOH	1.51	40.01	18* (see below)	59*
Ammonium hydroxide 58.6% NH_4OH	0.89	35.06	14.8	67

*The concentrated NaOH is described in the list of stock reagents below.

The normalities and volumes mentioned above are approximations only, with enough allowance for variations so that the solutions when prepared will have a concentration greater than 1 N (or 1 M in the case of H_3PO_4).

Carbonate-free concentrated NaOH :

Solid sodium hydroxide always contains some carbonate and readily takes up moisture and carbon dioxide from the air. A standard solution of this alkali cannot be made by dissolving a calculated quantity in the required amount of water. Fortunately,

sodium carbonate is practically insoluble in a saturated solution of sodium hydroxide. Therefore, a stock saturated solution of the alkali should always be kept on hand for the preparation of standard solutions.

Weigh hastily in a Pyrex beaker on a solution balance, 1100 g. (approximately) of the best quality (analytical grade) sodium hydroxide and place it in a two liter Pyrex beaker or flask. Add immediately one liter of distilled water and stir continuously until all the solid is dissolved. The solution becomes very hot. Allow to cool. After cooling, store in a Pyrex bottle, stoppered with a rubber stopper. Standardization of this concentrated solution is not advisable. It can be considered to be about 18 N in making up dilutions.

0.1 N Sodium hydroxide NaOH:

For each liter of 0.1 N NaOH to be made, use 5.9 ml. of the clear concentrated saturated NaOH (18 N). Dilute with freshly boiled and cooled distilled water to the required volume.

Standardization: Titrate with 0.1 N HCl whose normality is accurately known, using phenolphthalein as the indicator. Do not shake more than necessary to mix contents during the titrating. The solution may also be standardized against standard acid phthalate or acid iodate solutions or weighed out samples (see p. 22) with phenolphthalein as the indicator. In all acid-alkali titrations the alkali is placed in the buret, to avoid absorption of CO₂ from the air.

0.1 N Hydrochloric Acid HCl:

Measure about 17.5 ml. of the concentrated reagent grade hydrochloric acid (36% by weight) in a cylinder and pour the acid into a two liter volumetric flask, dilute to the mark and mix well. Determine the exact normality by titration against standard 0.1 N NaOH solution or by the use of sodium tetraborate (borax) either as a solution or as weighed-out samples. Tris (hydroxymethyl) amino methane may also be used as the primary standard.

0.1 N Potassium permanganate KMnO₄:

Although the pure crystals of potassium permanganate can be weighed accurately, it is not practical to use this salt as a primary standard because the solution tends to become weaker on standing. A very low concentration of organic substances in the distilled water used for the preparation of the solution slowly reduces the permanganate. The potassium permanganate solution is therefore prepared slightly stronger than is desired and allowed to stand undisturbed and tightly stoppered for a week before it is standardized with a sodium oxalate solution. In most cases it is advisable to standardize the permanganate each day before use.

Dissolve about 3.5 g. of KMnO_4 (analytical reagent) in about 1100 ml. of distilled water. Set aside in a tightly stoppered bottle for about one week. During this period the solution should not be disturbed so that any small quantity of manganese dioxide formed may settle to the bottom of the bottle. Carefully siphon off the clear supernatant liquid into a dark brown bottle.

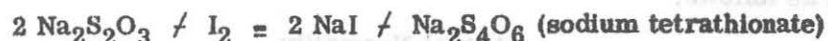
Standardization: Accurately pipet 20.00 ml. of 0.1 N sodium oxalate primary standard into a 500 ml. Erlenmeyer flask. Warm the container to $70-75^\circ\text{C}$. and titrate with the potassium permanganate solution until a faint pink color, that is given by the addition of a single drop, persists for one minute. If the titration is carried out slowly, the solution will become cool and the reaction slowed up. The solution in the flask should be kept at about 70°C . throughout the titration. The first few drops are decolorized very slowly but later the permanganate may be added rapidly. A check titration should agree with the first within 0.1 ml. Many analysts consider it unnecessary to adjust the strength of the permanganate solution to exactly 0.1 N, since a simple correction can be made in the calculations by using a "factor." See below. The permanganate solution should be kept in a dark brown bottle or one covered to keep out light. The titer of the solution may change somewhat, immediately after it has been freshly prepared but after standing for a week or so the strength is generally constant.

0.1 N Sodium Thiosulfate $\text{Na}_2\text{S}_2\text{O}_3$:

Weigh out about 25 g. reagent grade sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) and 5 g. of sodium tetraborate (borax) ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$). Transfer to a 1 liter flask and dissolve in about 800 ml. freshly boiled and cooled distilled water. Add about 7 ml. of iso-amyl alcohol and shake to dissolve. Dilute to about one liter. Transfer to a pyrex bottle and store for a few days before using.

Sodium thiosulfate solutions deteriorate on standing. This is stated to be due to decomposition of the salt by traces of CO_2 in the water, to bacterial decomposition, and to slow oxidation by dissolved oxygen. The purity and crystal water content are not sufficiently constant in the reagent grade to allow its use as a primary standard. The solution should always be made with boiled distilled water; the solution should be made alkaline and allowed to stand several days before standardization. The iso-amyl alcohol is added to minimize bacterial action.

Standardization: The thiosulfate is usually standardized by and used in reactions of iodimetry. The thiosulfate reacts with iodine as follows:



Thiosulfate solutions are best standardized with KIO_3 (potassium iodate) or $\text{KIO}_3 \cdot \text{HIO}_3$ (potassium bi-iodate) prepared as 0.1 N solutions. With a pipet measure 25.00 ml. of 0.1 N iodate or bi-iodate solution into a 250 ml. Erlenmeyer flask. Add

about 10 ml. of a freshly made 10% solution of KI (potassium iodide) and about 20 ml. of 1 N-hydrochloric acid (HCl). Iodine is liberated in this reaction thus;



Titrate the liberated iodine with thiosulfate from a 50 ml. buret. The thiosulfate is delivered into the iodate-potassium iodide-acid solution until the iodine color has faded to a very pale yellow. One ml. of starch test solution (see below) is added and the titration continued until the disappearance of the blue color.

Standard thiosulfate solutions weaker than 0.1 N are best made by diluting the 0.1 N standard with freshly boiled and cooled distilled water with an appropriate pipet and volumetric flask. If kept over a day these solutions should be checked with a diluted standard made from the potassium iodate primary standard. Store protected from the CO_2 of the air.

2/3 N Sulfuric Acid H_2SO_4

Weigh out 35 g. of concentrated sulfuric acid in a small tared beaker, dilute to 1 liter with distilled water and mix. Check by titration against standard NaOH using phenolphthalein as an indicator and adjust to 0.667 N.

1/12 N Sulfuric Acid H_2SO_4

Add 2.5 ml. of concentrated sulfuric acid to 1 liter of distilled water. Mix well and check by titration against 0.1 N NaOH so that 20 ml. of the acid requires 16.7 ml. of 0.1 N NaOH for neutralization using phenolphthalein.

2.5 N Sodium Hydroxide NaOH

Dilute 150 ml. of stock concentrated NaOH up to 1 liter with distilled water. Titrate against standard 0.1 N HCl and adjust to exactly 2.5 N.

The Use of a "Factor":

The calculations and adjustments of concentration are made using the usual formula; $V_1N_1 = V_2N_2$. In many cases, adjustment to exactly the nominal normality is not necessary. In this case, the use of a factor is convenient. The factor for any given solution is defined as follows:

$$\text{Factor} = \frac{\text{Actual Normality}}{\text{Nominal Normality}}$$

Example: A solution of NaOH is made up to be close to 0.1 N. Titration shows it to be 0.0987 N. The factor is then 0.9870. If the titration had shown it to be 0.1032 N the factor would have been 1.032.

Starch Test Solutions:

1. Triturate (grind) 1 g. of arrowroot starch with 10 ml. of cool distilled water and pour slowly with constant stirring into 200 ml. of boiling distilled water. Boil the mixture until a thin, translucent fluid is obtained. Allow to settle and use only the clear, supernatant liquid. Longer boiling than necessary renders the test solution less sensitive. The solution must be freshly prepared.

2. Another popular method for making a solution of starch is to suspend 100 g. of pure cornstarch (Kingsford's) in approximately one liter of 0.01 N hydrochloric acid with shaking at frequent intervals for one hour. Decant the supernatant liquid after sedimentation. Wash twice, with one liter each time, with 0.05 per cent sodium chloride solution. Spread out and allow to dry in the air. Thoroughly grind 15 g. of this washed starch in a mortar with 50 ml. of distilled water and pour into 900 ml. of boiling distilled water. Boil for 1/2 to 1 minute without agitation. Cover the mouth of the flask with a beaker and set in a boiling water bath for 15 to 30 minutes. A few drops of this solution are sufficient for a titration.

3. A one per cent solution of a high grade soluble starch (Merck's soluble starch) in cold distilled water has been also successfully employed for iodometric titrations. Most starch solutions deteriorate quickly on standing with the growth of molds. The solution of Pincussen, which is made by dissolving 1 g. of soluble starch in 10 ml. of boiling distilled water and adding 90 ml. of saturated sodium chloride solution, keeps satisfactorily.

VOLUMETRIC CALCULATIONS

A. Solid Standard Standardization of NaOH

Samples of potassium acid phthalate ($\text{KHC}_8\text{H}_4\text{O}_4$) M.W. 204.22, are weighed out and dissolved in about 25 ml. of distilled water and titrated by the unknown sodium hydroxide solution, using phenolphthalein as an indicator. Three samples gave the following results:

Sample	Weight (g.)	ml. NaOH	*	meq. total	meq./ml. NaOH=(N) (calc.)
1	0.8436	41.24	*	4.131	0.1002
2	0.8978	43.91	*	4.396	0.1001
3	0.8621	42.05	*	4.221	0.1004

1. The weight of the samples divided by the molecular weight of potassium acid phthalate multiplied by 1000 gives the total meq. of acid (and of alkali),

2. Since this number of milliequivalents is present in the indicated volumes of NaOH, division of total meq. present by number of milliliters of NaOH gives meq./ml. or normality of the NaOH.

B. To prepare a Normal Solution from a Concentrated Acid Solution:

1. Find the normality of the concentrated acid.

- Multiply the specific gravity by the assay (% by weight) to get g./ml.
- Multiply by 1000 to get g./liter.
- Divide g./liter by the equivalent weight to give meq./liter which is by definition the normality of the concentrated acid.

2. Use the formula $V_1N_1 = V_2N_2$

Example: Make 3 liters of 0.16 N HNO_3 from concentrated acid.

1. Find the normality of the concentrated acid (69% by weight).

- 1.48 (specific gravity) $\times 0.69$ (assay) = 1.0212 g./ml.
- $1.0212 \times 1000 = 1021.2$ g./liter
- $1021.2/63$ (equivalent weight) = 16.2 equivalents/liter = normality.

$$2. 3000 \times 0.16 = V_2 \times 16.2$$

$$\frac{3000 \times 0.16}{16.2} = 29.6 \text{ ml. concentrated acid to be diluted to 3 liters.}$$

C. To Prepare a Normal Solution from a Percentage Solution:

1. Find the g./liter in a 1 N solution (one equivalent weight).
2. Find the g./liter in the % solution.
3. Find the N of the % solution by division of Step 2/Step 1.
4. $V_1 N_1 = V_2 N_2$

Example: Make 2 liters of 3 N HCl from 37% HCl (concentrated acid)

1. 1 N HCl = 36.5 g./liter.
2. 37% HCl has a specific gravity of 1.19.
 $1.19 \times 0.37 \times 1000 = 440 \text{ g./liter.}$
3. $440/36.5 = 12.08 \text{ N.}$
4. $2000 \text{ ml.} \times 3 \text{ N} = V_2 \times 12.08 \text{ N.}$

$$\frac{2000 \times 3}{12.08} = 497 \text{ ml. concentrated HCl (37\%) to be diluted to 2000 ml. to obtain 3 N HCl}$$

D. To Prepare a Percentage Solution from a Normal Solution

1. Determine the equivalent weight.
2. Find the g./liter in the percentage solution.
3. Find the N of the percentage solution by dividing Step 2/Step 1.
4. $V_1 N_1 = V_2 N_2$.

Example: Make 2 liters of 5% HCl from 3 N HCl.

1. 36.5 g./liter in 1 N HCl (one equivalent weight).
2. 5% HCl is 5 g./100 ml. or 50 g./liter (W/V).

3. $50/36.5 = 1.37$ (normality of 5% HCl).

4. $2000 \times 1.37 = V_2 \times 3 \text{ N}$

$$\frac{2000 \times 1.37}{3} = 913 \text{ ml. } 3 \text{ N HCl to be diluted to 2 liters to obtain 5\% HCl.}$$

E. To Prepare a Percentage Solution from a Percentage Solution:

1. Find g./ml. contained in the given solution.
2. Find total g. needed for total amount of new solution.
3. Then $\frac{\text{total g. needed}}{\text{g./ml. given}} = \text{ml. of given \% to be diluted to the required total volume.}$

Example: Make 3 liters of 7% sulfuric acid from 15%

1. $15\% = 15 \text{ g./100 ml.} = 0.15 \text{ g./ml.}$
2. $7\% = 7 \text{ g./100 ml.} = 70 \text{ g./liter} = 210 \text{ g./3 liters.}$
3. $210/0.15 = 1400 \text{ ml. of 15\% to be diluted to 3 liters to obtain 7\%.}$

An alternative procedure is to use the relationship $\%_1 V_1 = \%_2 V_2$

In the above example $15 \times V_1 = 7 \times 3000$

$$V_1 = \frac{7 \times 3000}{15} = 1400 \text{ ml.}$$

F. To Calculate Normality of a Percentage Solution:

1. Find the molecular weight of the substance.
2. Find g./liter of a 1 N solution.
3. Find the percentage of a 1 N solution.
4. Divide given % by 1 N %. Step 2 divided by Step 3.

Example: Find the normality of 10% H_2SO_4 .

1. Molecular weight $\text{H}_2\text{SO}_4 = 98 \text{ g.} = 2 \text{ equivalents.}$
2. 1 N H_2SO_4 contains 49 g./liter.

voltage developed is proportional to the ratios of the concentration of hydrogen ions inside and outside of the glass membrane.

Commercial glass electrode pH meters read directly in pH units. They should be calibrated just before use by the aid of at least two standard buffer solutions differing by at least 2 pH units. See pH standards p. 24.

For best results follow the instructions given by the manufacturer of the particular instrument used. Most directions suggest using only one standard buffer. The use of two will detect errors due to cracked or otherwise faulty electrodes; errors not easily shown up by single point calibration.

pK_a Values for Some Acids Suitable for Buffers

Name of Acid	Formula	pK_a	Buffers at pH
Pyrophosphoric	$H_4P_2O_7$	(1) 0.854	1
"	"	(2) 1.959	2
Phosphoric	H_3PO_4	(1) 1.959	2
Glycine (as base)	NH_2CH_2COOH	11.647 (pK_b)	2.3
Phthalic	$C_6H_4(COOH)_2$	(1) 2.90	3
Tartaric	$HOOC(CHOH)_2COOH$	(1) 2.96	3
Citric	$(HOOCCH_2)_2C-OH$ COOH	(1) 3.1	3
Lactic	$CH_3CHOHCOOH$	3.98	4.2
Barbituric	$NHCONHCOCH_2CO$	3.98	4.2
Tartaric	see above	(2) 4.16	4.7
Acetic	CH_3COOH	4.73	4.7
Citric	see above	(2) 4.75	4.7
Citric	see above	(3) 5.40	5.4
Phthalic	see above	(2) 5.51	5.5
Carbonic	H_2CO_3	(1) 6.53	6.5
Pyrophosphoric	see above	(3) 6.54	6.5
Phosphoric	see above	(2) 6.7	6.7
Pyrophosphoric	see above	(4) 8.44	8.4
Boric	H_3BO_3	(3) 9.20	9.2
Glycine (as acid)	see above	9.78	9.8
Phosphoric	see above	(3) 12.44	12.4
Salicylic	HOC_6H_4COOH	(2) 13.0	13.

COLORIMETRIC TECHNIQUES

The method of analysis known as colorimetry has made possible the development of methods for analysis of small amounts of blood containing very minute amounts of material. This in turn has led to the present widespread knowledge of the variations in blood constituents in health and disease. This knowledge is, of course, not complete and new methods and new applications continue to be made. Outside the scope of this manual is the extensive literature of what is called "ultra-micro analysis," since in most cases a few milliliters of blood will suffice using the methods in this manual. In pediatric work, infants and children cannot furnish blood as easily or in such large amounts, so methods of analysis based on the volumes obtained by heel or finger puncture have been developed. Some of these are included in this manual.

Colorimetry depends on the quantitative comparison of the amount of color developed in unknown solutions with the amount of color developed in solutions having a known amount of pure substance present. The comparison is most often made visually, matching unknown tubes with standard tubes, or matching visual fields in a special apparatus such as a Duboscq colorimeter.

This measurement technique is based on Beer's Law (sometimes called Lambert-Beer's or Bouguer-Beer's) which states (briefly and approximately) that the amount of light absorbed by a colored solution is proportional to the concentration of colored material present. Beer's Law applies in a practical way only to solutions with a relatively small range of concentration, and only when the incident light is "mono-chromatic;" (actually, a narrow band of wavelengths). These requirements are only relative and to some extent interdependent. The optimal conditions for colorimetry are best determined individually for each determination.

Colorimetric measuring apparatus:

Block comparators: The block or rack comparator is used in situations in which simplicity and speed (for example: bed-side analysis) require the sacrifice of accuracy. This method uses a series of graded standards (usually in sealed tubes) against which the developed color or turbidity is compared. An example of a method in which block comparison is commonly used is the ICTERUS INDEX, p. 187.

Dilution Colorimetry: This type of comparison colorimetry uses a single, fixed standard and the unknown is diluted until the standard is matched. The Sahli hemoglobin method is an example of this type of analysis. The fixed standard in this procedure may be a permanent standard such as a colored glass disc.

Comparison Colorimeter: This is the method commonly employed for visual colorimetry. The apparatus used may vary in the details but all have some arrangement whereby the thickness (or depth) of a layer of the standard and the unknown colored

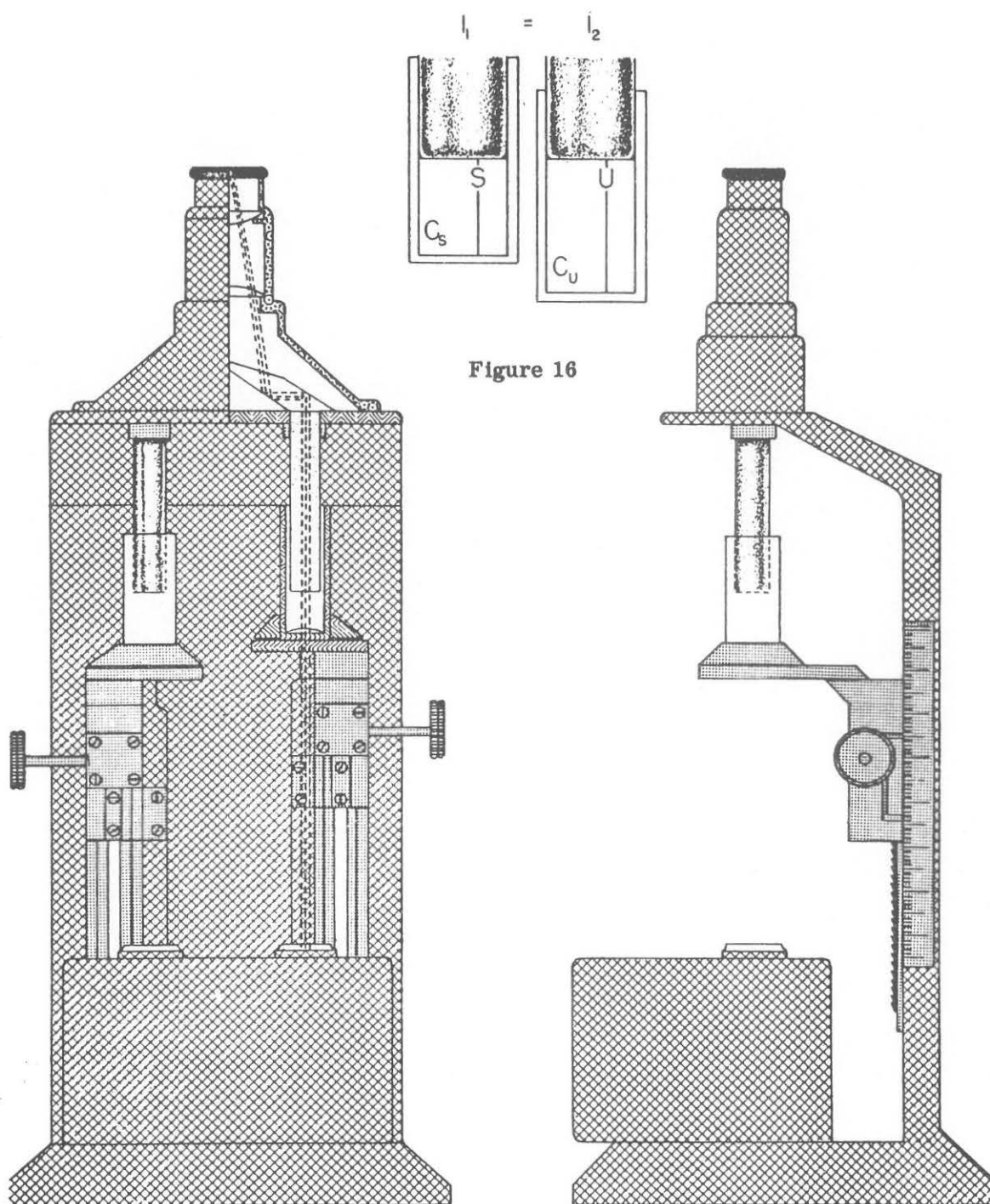


Figure 16

Figure 15. Duboscq colorimeter



Fig. 13. Improved color camera

solutions may both be varied and we measure the depths of solutions that give equal light absorption. The Duboscq colorimeter is of this type and is quite convenient for this purpose (see Fig. 15, p. 48a).

Beer's Law:

Colorimetry is based upon Beer's Law, which states that light in passing through a colored medium is absorbed in direct proportion to the concentration of the colored substance. Thus, the intensity of the observed color is directly proportional to the concentration of pigment in solution and directly proportional to the depth of the observed layer; and thus is actually proportional to the number of colored particles in the light path.

For example: It would require a 20 mm. depth of a 1% solution of a dye to exhibit the same intensity of color as 10 mm. of a 2% solution of the dye. Thus:

$$(1) \quad R_1 (20 \text{ mm.}) \times C_1 (1\%) = R_2 (10 \text{ mm.}) \times C_2 (2\%)$$

$$\text{or} \quad 20 \times 1 = 10 \times 2 \quad (\text{see Fig. 16, p. 48a.})$$

Obviously, if the light transmitted by a solution of unknown strength is compared with the light transmitted by a solution of a known strength, the concentration of the unknown can be calculated by means of this formula. Beer's Law, (for colorimeters) stated mathematically, is:

$$(2) \quad R_1 C_1 = R_2 C_2$$

$$\text{or} \quad C_1 / C_2 = R_2 / R_1$$

Here R_1 and R_2 are the readings (depths) of solutions of unknown and standard in the colorimeter cups when their transmissions match by visual observation. C_1 and C_2 are their respective concentrations. This may be rewritten to solve for C_1 , thus:

$$(3) \quad C_1 = (R_2 / R_1) \times C_2$$

C_1 in this equation represents the concentration in the final colored unknown solution. If only a small amount of material is used in this test and it is desired to express the concentration in terms of 100 ml., a volume correction factor (k) is introduced into the equation:

$$(4) \quad C_1 \times (k) = C_u = (R_s / R_u) \times C_s \times (100 / V)$$

In this equation C_u is expressed (for example) in milligrams per 100 ml. of serum (mg.%); C_s is expressed in mg. of standard substance in the final volume of colored standard solution; and V represents the actual volume of original sample. For

example, if 1 ml. of a 1:10 protein-free filtrate is used in the analysis, V would be equal to 0.1 ml. The above formula (4) assumes that the final volumes of the colorimetric solutions of the unknown and the standard are the same. In some determinations this may be not the case. A dilution factor (V_u/V_s) is accordingly introduced to correct for this difference. The final equation upon which all colorimetric calculations in blood and urine analysis are based, thus becomes:

$$(5) \quad C_u = (R_s/R_u) \times C_s \times (100/V) \times (V_u/V_s)$$

in which V_u and V_s are respectively, the final volume of the unknown and the standard colored solutions. 100 ml. here is the reference volume, and this is the value often used for blood analysis. For urine it may be replaced by the 24-hour urine volume or one liter (1000 ml.) depending on the particular application.

Colorimetric calculations are greatly simplified by choosing values of C_s , V , and the colorimeter settings with that end in view. When the standard solution is set at a certain depth (R_s) the final calculation usually involves dividing the reading of the unknown into some simple factor which however, cannot be done mentally. Instead of using elaborate tables or calculating devices for this purpose, the expedient of setting the unknown at a given depth and reading the standard, enables one to complete the calculation mentally by a simple multiplication. For example, in a certain uric acid method, setting the standard at 20 mm. requires the division of the reading of the unknown into 80; while setting the unknown at 20 mm. requires only the multiplication of the reading of the standard by 0.2.

The standards employed, which are solutions of known concentration, may be of several types, their selection depending upon the nature and stability of the color developed in the test. Theoretically, the most desirable standard is one prepared by treating a known amount of the substance being analyzed with the reagent at the time of preparation of the unknown. In this way, all of the slight variations of temperature, period of heating, and standing, etc., are equalized. It is occasionally practical to use an artificial standard made of more stable materials, such as a solution of one or more dyes, or a solution of inorganic colored salts, or tinted glass plates. When using artificial standards it is necessary to check their equivalence frequently against the true color developed by known concentrations of the substance in question. In the case of colored glass plates used as artificial standards, the advantage of permanence may be offset by an imperfect color match. The colored plates should be standardized by spectrophotometric measurement to determine the plate equivalence in one's own laboratory.

The colorimeter makes possible the quantitative comparison of the unknown and the standard solutions. It is a precise optical device by which the intensities of color in two solutions can be compared. If the intensity of color is quantitatively related to the concentration of a substance, colorimetry may be employed for its quantitative estimation. The plunger type of instrument is best suited to general use in biochemistry. This instrument has two plungers which dip into the solutions contained in cups, and whose

relative positions may be regulated by racks and pinions. Light of uniform intensity is passed in parallel rays through the two fluids to be compared. The depth of one or both columns of fluid through which the light passes can be regulated at will by a vertical motion of the cups. The depths are so adjusted that the colors from the two solutions which are seen in a bisected visible field, appear equal.

The colorimeter cups are of various types and sizes. The bottom of each cup is formed by a plate of clear optical quality glass. The tops may be flared out to serve as reservoirs for the solution displaced by the plungers when the cups are raised. These cups are optically perfect and such substitutes as small beakers cannot be used without greatly decreasing the accuracy which can be attained. Millimeter scales are arranged in connection with the cup supports so that they can measure the depth of fluid between the lower surface of the plungers and the bottoms of the cups. With the aid of verniers, measurement is precise to within 0.1 mm.

The visual field of the instrument is a circle divided into two halves. The dividing line is formed by the ends of the prisms which transmit the light that has passed through the solutions. The optical path is arranged so that the light passing through the right hand cup appears on the left field and vice versa. When the two halves of the field show identical color intensity, the conditions stated above in equations (1) and (2) are satisfied.

Testing the Colorimeter:

Before a colorimeter is used each day and at frequent intervals during the day, it should be tested with regard to the zero points, the equality of illumination, and the equality of the quality of the transmitted color.

Zero Point Test: The empty cups are first placed in position on the apparatus and carefully raised until the plungers come into contact with the bottom of the cups. Then the readings should be zero. If this is not the case, readings must be corrected for the zero point error.

Equality of Illumination: Care must be taken that the illumination of the two fields is equal. Equal illumination can be secured best by using an even, ample source of light, preferably diffuse daylight or white artificial light. There are numerous satisfactory lamps on the market. Some colorimeters are provided with a light in the base, thus obviating the necessity of an outside source.

Portions of the same colored fluid, usually the standard solution, are placed in each cup, rinsing the cups and the plungers, and taking care to avoid bubbles under the plungers. The cups are then set to have identical fluid depths and the light source adjusted until the two halves of the visual field appear identical. After this has been done, colorimetric comparison may be carried out as outlined below. The colorimeter and its light source should not be moved or altered after this light adjustment has been made. If a change does occur the light balancing should be rechecked before colorimetric comparisons are made.

Colorimetric Comparison:

There should always be enough fluid to cover the lower ends of the plungers within the practical range of motion of the cups during a determination but the cups should never be so full that the liquid is driven over the top when the cups are raised to the reading point.

After the above tests are completed, the standard solution is allowed to remain in one of the cups and the unknown is placed in the other, rinsing the cup and the plunger with portions of the unknown. The depth of the standard is set at some convenient point, usually about 10, 15, or 20 mm., and the height of the cup of unknown is adjusted until the two fields match. Several such adjustments are made, approaching the match point from both sides, and the average of such readings obtained is used for calculation. It is sometimes more convenient to set the depth of the unknown solution at a pre-determined point and then to adjust the depth of the standard solution to obtain the match.

The stationary scale is graduated in cm. and divided into mm. All readings are made in mm., e.g., 2 cm. is read 20 mm. The point at which the zero line of the movable scale would intersect the stationary one is taken as the reading. Decimal fractions are read at the point where two lines of the vernier and movable scales approximate most closely.

Calculations are made according to Beer's Law which has been discussed above.

Limits of Accuracy - Sources of Error

Few observers can, with the usual colorimetric instruments, match colors with an error of less than 1%; for most analysts the error is probably nearer to 2 or 3%. The accuracy is greatly influenced by characteristics of the individual. Some persons are incapable of matching colors: others have difficulty only with certain colors. Adjustments should be made rapidly in order to avoid tiring the eyes.

The range within which depth of solution is proportional to intensity of color is quite limited. Standard and unknown can seldom be permitted to differ from one another by more than a factor of 2. If the difference is greater than this another standard must be used or the test must be repeated with a lower concentration (or higher, if necessary) of the unknown.

PHOTOMETRIC TECHNIQUES

This procedure and technique differs from the method just described in two respects: (1) The depth of fluid examined is not varied and then measured, but on the contrary, the depth is kept constant and the same for both standard and the unknown solutions. (2) The measurement which is made is not of depths giving equal light absorption, but an actual determination of the amount of light transmitted by the standard and by the unknown solutions. This measurement is made possible by use of a photo-electric cell, which generates a current proportional to the amount of light striking the photo-cell. The current is then indicated on the scale of a sensitive galvanometer.

The relationship between the concentration of color in a solution and the amount of light transmitted through the solution can be treated in a mathematically rigorous fashion and this has been done in some of the reference books to which your attention has been directed. See p. 340. The biochemist and clinical technologist are usually interested in the simple comparison (in a quantitative way) of the amount of color developed by one or more unknown solutions with the amount developed by a solution containing a known amount of a standard substance.

A diagrammatic outline of a simple filter photometer of a type commonly used in a laboratory is shown in Fig. 17, p. 53a. The white light from the bulb A passes through the color filter D. The light now passes through the test tube cuvet B containing the colored solution C. The emergent light strikes the photo-cell E, causing the generation of a current which is measured by the galvanometer G. The colored light filter may occupy the position shown or may be inserted between the cuvet B and photo-cell E.

Briefly, the procedure in photometric analysis consists of the following steps:

(1) Pure solvent (usually water) or a "reagent blank," is used in a test tube cuvet. The cuvet is placed in position in the photometer and the rheostats R are adjusted so as to produce a reading of 100 on the galvanometer. The "blank" tube is then removed.

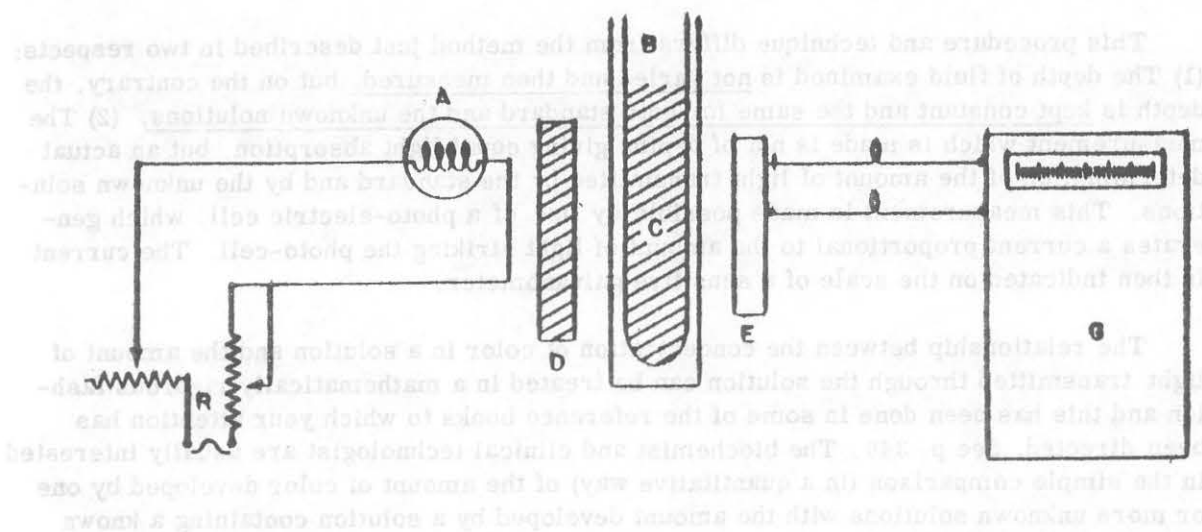
(2) Without readjusting the rheostats R, the test tube containing the standard solution is placed in the photometer and the galvanometer deflection is read and recorded. The "standard" tube is then removed.

(3) Again without readjusting the rheostats, the test tube containing the unknown solution is placed in the photometer and the galvanometer is read and the result recorded.

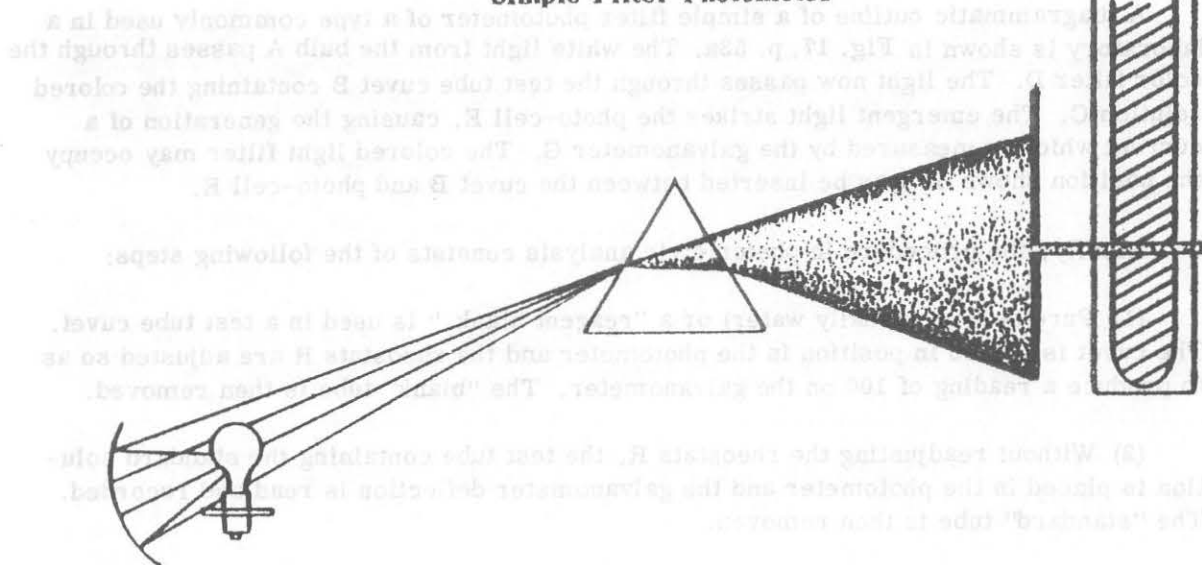
We have now a set of data consisting of the following:

$$(1) T_b = 100; (2) T_s = X; (3) T_u = Y$$

where T_b is the per cent transmittance of the blank (arbitrarily 100); T_s is the per cent transmittance of the standard solution read from the galvanometer; and T_u is the transmittance of the unknown solution, read from the galvanometer.



Simple Filter Photometer



Spectrophotometer (up to the photocell)

Figure 17. Two types of photometers

Filter Selection:

The selection of the proper filter (D in the diagram) to use in a given analysis depends on the color which is being measured. Ideally, the filter should allow those wavelengths of light to pass which the colored solution most strongly absorbs. Thus a blue solution is blue because it absorbs most of the light in the red end of the visible spectrum, and transmits most of the light in the blue end of the spectrum as diagrammed in Fig. 19, p. 54a. (A, B, C, D.)

For the most sensitive analysis of this blue solution we would choose a filter which transmits light at the red end of the visible spectrum, i.e., a red filter (indicated by the area with diagonal lines). It is never possible in practice to obtain filters which transmit exactly as indicated in the diagram. Instead the true graph of the transmittance (and absorbance) of a red filter would appear as shown by the curved solid line (X, Y, Z).

Some filters have narrower transmittance bands and some have wider bands than this. In order for the solution to act in accordance with Beer's Law it is necessary to have the filter transmit only those wavelengths of light which are absorbed maximally by the solution being tested. That is, those wavelengths on the flat part of the curve (C, D). This condition results in (a) the greatest sensitivity and (b) the narrowest range for any given method.

In some instances it is desirable to extend the range at the expense of sensitivity and to some extent at the expense of linearity. This can be done by choosing a filter transmitting wavelengths between B and C in the example above. Further details regarding the choice of a filter can be found in the reference books listed.

Reagent Blanks:

Since we are not working with ideal conditions and since reagents are not always pure, we often have a small amount of color or turbidity resulting in light absorption even when the reaction is carried out on distilled water or some other pure solvent. Such a preparation is called the "reagent blank." There are two ways in which the blank can be utilized to correct for the impurities present and these will be discussed under GENERAL PHOTOMETRIC TECHNIQUE below.

Beer's Law for Photometry:

Let us assume that we have prepared a series of standards varying in concentration along with a "reagent blank" and have recorded the corresponding galvanometer readings (which represent the transmittance in per cent), which are given here:

Relative concentration	Transmittance %	$2 - \log T = (D)$
0	100.0	0.0000
1	80.2	0.096
2	64.3	0.192
3	51.5	0.288
4	41.3	0.384
5	33.1	0.480
8	17.1	0.767
10	11.0	0.959

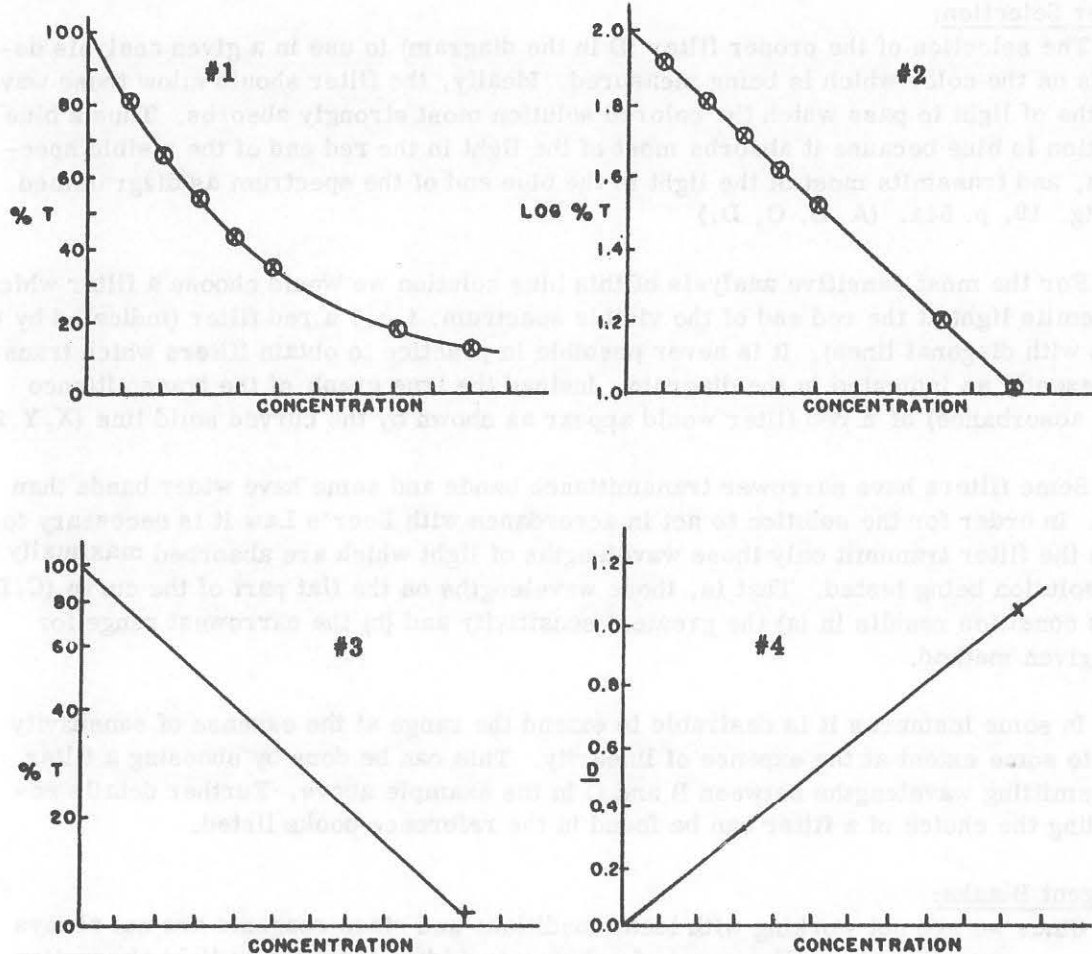


Figure 18. Types of photometric calibration graphs.

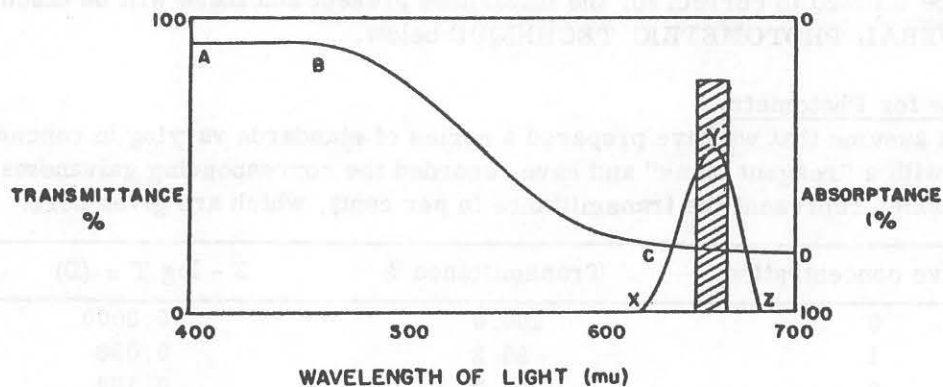


Figure 19. Absorbance and Transmittance spectra.

There are a number of ways in which the relationship between concentration of solute and galvanometer readings can be expressed. See Fig. 18, p. 54a. We note that in graph #1 (per cent transmittance vs. concentration) the result is a curved line. This type of relationship is difficult to use routinely because it makes interpolation and extrapolation difficult and inaccurate. Graph #2 (log per cent transmittance vs. concentration) results in a straight line with a negative slope. Graph #3 (per cent transmittance vs. concentration) results in a straight line with negative slope if a special graph paper (semi-log) (ordinate plotted logarithmically and the abscissa plotted linearly) is used. Graph #4 is the easiest to use. It is a plot of the optical density (D) or $(2 - \log \%T)$ vs. concentration. This plot results in a straight line with a positive slope. That is, an increase in concentration results in an increase in optical density.

All of these graphs are expressions of Beer's Law, which can be expressed mathematically (for photometric analysis) as:

$$(1) \quad \log (I_c/I_o) = -kc$$

where I_c is the intensity of transmitted light;

I_o is the intensity of incident light;

c is the concentration of the colored substance.

This equation, stated in words, is that the logarithm of the ratio of the intensity of transmitted light to the intensity of the incident light is inversely proportional to the concentration of colored substance. k is a constant whose actual value depends on the nature of the colored solution, the filter used, the depth of the solution, etc.

In the experiment above, the incident light (the light transmitted in the absence of color in the solution -- the "blank") was arbitrarily set at 100% and therefore:

$$(2) \quad \log (I_c/I_o) = \log (I_c/100)$$

I_c can now be termed the per cent transmittance and may be designated with the symbols T or G (for transmittance or galvanometer reading).

Rearranging equation (2)

$$\begin{aligned} \log I_c - \log 100 &= -kc \\ \text{or } \log 100 - \log I_c &= kc \\ \text{or } 2 - \log I_c &= kc \end{aligned}$$

If $(2 - \log I_c)$ be given the name "optical density" and the symbol "D" then:

$$D = kc$$

It is obvious that if experimentally a plot of D (optical density) vs. C. (concentration) results in a straight line - usually passing through the origin (O, O), the results are in accordance with Beer's Law. Not all reactions giving colors will act this way, although a judicious selection of filters and/or the use of the more selective instruments such as the prism or grating spectrophotometer usually do result in a straight line graph (if the reaction conditions are correct).

In cases of failure to obey the law, it will be necessary to use the plot of D vs. C (which will be a curved line in these cases), or to prepare a table derived from such a plot, to estimate concentration from the optical density.

General Photometric Technique:

1. Selection of filter: Selection will depend on the color developed in the determination. The wrong filter will give incorrect results.

2. Warm up: Allow the instrument to come to a "steady-state." This will take 15 to 20 minutes.

3. Adjust galvanometer to read zero: Adjust the zero point reading when no light is striking the photoelectric cell. This is very important because the 100% setting is not really 100% and no other reading will be correct unless the zero setting has been accurately made.

4. Set the "blank" to read 100: Insert the cuvet or selected test tube containing the "blank" solution (read further below) and adjust the light intensity so that the galvanometer reads 100%.

5. Read the standard: Remove the blank tube, insert an identical cuvet or test tube containing the standard and read its transmittance T_g , in per cent.

6. Read the unknown: Remove the standard tube, insert an identical tube or cuvet containing the unknown solution, and obtain its transmittance T_u in per cent.

7. If the photometer was adjusted to read 100 using a pure solvent as the "blank," the "reagent blank" must now be read and its transmittance recorded as T_b in per cent.

"Blank" definitions:

It will be noted that the term "blank" has been used in several different ways in the above discussion. For the purpose of clarification the following definitions should be carefully noted.

"Optical Blank" is the solution used to set the photometer at 100 per cent transmittance.

"Reagent Blank" is the solution obtained when the colorimetric reaction is carried out on pure water rather than on a solution containing the substance analyzed for.

"Zero-time Blank" is the solution obtained when no time at all is allowed for a reaction whose optical density increases with time. This is commonly used only in cases of estimations of enzyme activity, when there is present in the serum some constituent which is being formed from a substrate by enzymatic activity.

It should be noted that the reagent blank and the zero-time blank can be utilized as optical blanks. It is of some advantage to do so, because by such a procedure, the blank value is automatically subtracted from the unknown and avoids an extra calculation. See below for the details of such calculations.

Photometric Calculations:

As in visual colorimetry we are determining the ratio of the concentration of color in the unknown as compared to that in a standard and therefore the general formula is very similar, remembering that the transmittance must be converted to optical density before inserting in the formula.

Visual colorimetry formula

$$C_u = (R_s/R_u)C_s$$

Photometry Formula

$$C_u = (D_u/D_s)C_s$$

Also note that the readings (R) in colorimetry are inversely related to the concentration while in photometry the densities (D) are directly related to concentration.

The complete formula for photometric calculation is analogous to the colorimetric formula p. 49.

$$(3) \quad C_u = (D_u/D_s) \times C_s \times (100/V) \times (V_u/V_s)$$

Since in most photometric methods, V_u/V_s is 1, the last term usually cancels out.

Whenever blanks with an optical density greater than 0, ($T = < 100$) are obtained, (such as reagent blanks or zero-time blanks read against a pure solvent optical blank) the optical density of the blank is subtracted from the unknown and standard densities before inserting the values into the formula.

When it has been shown that a result is readily duplicable for long periods of time, it is possible but not recommended to dispense with the use of standards and to use the relationship, $D = kc$, where k is the constant relating density and concentration by a combination of factors such as the reaction characteristics and the characteristics of the color and the filter used, etc. Thus, in use, k is determined by the use of standard solutions and thereafter the density values of the unknowns is multiplied by k to obtain the final result, say in mg. per 100 ml. of blood.

This procedure is somewhat dangerous and should not be resorted to unless absolutely necessary. It is used in cases in which scarcity or instability of the standard material limits the supply as for example a pure bilirubin, urobilinogen, etc.

Some photometers, such as the Klett, have a scale reading directly in optical density units. Unless this is the case, the log of the ratio must be calculated for I_c/I_o or a table of D vs. T values may be used. See Appendix for such a table.

In the foregoing photometry discussion, it was assumed that a constant depth of solution was being used. This laboratory uses especially selected test tubes as absorption cells in most cases. Although these do not have a uniform depth of solution for all parts of the tube, still the effective depth is the same for the matched tubes. Since test tubes inevitably are somewhat oval rather than round, it is imperative that they be positioned in exactly the same way each time they are used as well as when they are calibrated. See p. 330 for a note on the Calibration of Test Tube Cuvets. Calibration can be avoided if the same test tube (in the same position) is used for the reading of the standard and for the unknowns as well as for the blank setting or reading. However, in some analyses, this procedure is impractical.

Photometric Instrumentation:

In the discussion above, a simple single photo-cell photometer is described. There are, however, a large variety of types of instrument available on the market. They may be classified as follows:

The essential parts of any photometer are:

- (1) A source of light equipped with
 - (a) a means of adjusting the intensity of the light falling on the solution and
 - (b) a means of stabilizing the intensity of the light so that it remains constant for long periods. Since the emissivity of a tungsten lamp is proportional to the fourth power of the absolute temperature, the power supplied to the lamp must be very closely regulated.
- (2) A means of wave-length isolation which may be by:
 - (a) Color filters - plates of colored glass with definite absorption characteristics.
 - (b) Interference filters - plates of glass with half-wavelength reflection.
 - (c) Prism or gratings - used in spectrophotometers. These can be rotated to select a given narrow band of wavelengths.

The usual way of expressing the band width of a filter or other device for isolating a band of wavelengths is to state the band width between the points giving 50% of the transmittance of the peak. Using this notation, Corning glass filters have a band width of 50 to 150 m μ ; some interference filters have a width of 10 to 40 m μ ; for the Coleman spectrophotometer the width is about 35 m μ ; for the Bausch and Lomb Spectronic 20 - about 20 m μ ; and for some more precise instruments as small as 1.0 or 0.5 m μ depending on the wavelength. These instruments may be equipped with quartz optics to enable their use in the ultra-violet region of the spectrum.

- (3) Cuvets - these may be
 - (a) very precise square cuvetts
 - (b) especially selected test tubes - less accurate, more convenient
- (4) Photo-cell(s) may be of two types
 - (a) Barrier layer cells - these generate their own current but require a sensitive galvanometer since the current cannot be readily amplified.
 - (b) Photo-emissive cells - which change resistance upon exposure to light and require an external current source. The current in this case may be readily amplified, thus a less sensitive galvanometer is required.

The available instruments may also be classified as single cell or double cell instruments. The single cell type is the simple type described above, p. 53. The double cell type consists of two closely matched photocells which can be balanced against each other so that no current passes when the reading is made. The Klett-Summerson instrument is of this type. The reading is made in optical density units from a dial connected to the balancing rheostat circuit.

Flame Photometry:

This is a very specialized application of photometry in which a finely divided aerosol spray of sample is passed into a flame. The heat excites the elements present so that they emit light. This light is filtered by use of glass color filters or by prism or grating arrangements and the wavelengths of interest are allowed to fall on a photocell. The light produced is proportional within narrow limits to the amount of a particular element present.

This method is commonly used for sodium and potassium in many laboratories. To compensate for some of the many variables, the double cell method is used in some of the instruments.

In the double-cell (or internal-standard) method, one cell and filter combination is responsive to say sodium emission, and another cell-filter combination is responsive to lithium emission. Since lithium does not exist in biological fluids it can be added to the same concentration in known and unknown solutions. When this is done the readings of the instrument are responsive only to the ratio of sodium to lithium and since any factor decreasing sodium emission will also decrease lithium emission, the reading is quite stable. See further under Na and K.

In the use of all these instruments it should be remembered that ordinary white light is actually made up of all the colors in the rainbow. By filters or by gratings or prisms white sunlight may be separated into its component colors. One thing these colors all have in common is that they travel in waves. The important difference between the colors is that each one has a different wavelength (the distance between the crest of one wave and the crest of the next). When all the colors in white light are spread out and arranged by wavelength (as may be done in a rainbow or by a prism), they make up a continuous band. At one end of the band is violet, with a wavelength of 400 millimicrons (μ) which is about 16 millionths of an inch. Next in order come indigo, blue, green, yellow, orange, and finally red light, whose wavelength is comparatively long (700 μ).

These wavelengths from 400 to 700 μ (the so-called "visible" range) are the only ones the human eye can see. Light with wavelengths outside of this region does not promote a visual response of color and brightness in the human eye. At wavelengths just below our area of visibility lies the ultraviolet region. Above the red area of light lies the infrared region.

Chemical analysis may be done by use of a spectrophotometer because the atoms in a molecule resonate to certain wavelengths of ultraviolet, visible, or infrared radiation. The wavelengths which cause this vibration are absorbed; the rest of the wavelengths are transmitted through the sample. Now if we make a graph showing how much of each wavelength a particular chemical compound absorbs, we have its "absorption spectrum." Fig. 17, p. 53a shows how the spectrophotometer measures the amount of a particular color (wavelength) of light absorbed or transmitted by the sample substance. By making a series of such measurements and using different wavelengths of light, the analyst can draw a curve which shows the exact location and degree of the absorptions of the sample over a wide range of wavelengths. This curve is the "absorption spectrum."

If a series of measurements are made with different concentration at a certain specified wavelength the results obtained are called a "calibration curve". This can be used in quantitative analysis as discussed above on page 55.

GASOMETRIC ANALYSIS

In the study of gases and gas volumes we must constantly be aware of the effects of temperature and pressure and their changes from one determination to the next. The volume of any gas depends upon the temperature and pressure to which it is subjected and the volume of a gas may vary widely with changes of temperature or pressure. It is necessary, therefore, in order to compare a volume of a gas measured under one temperature and/or pressure with a volume of gas measured under a second temperature and/or pressure, to have a set of uniform correction factors.

The Standard Gas Conditions are as follows:

1. Atmospheric pressure at sea level (760 mm. column of Hg).
2. The temperature of water at its freezing point (0° centigrade or 273° absolute).

Gasometric methods are those which depend upon the measurement of a gas as the final step in the analysis. The measurement must take into consideration the so-called gas laws.

The gas laws governing the behavior of the gases are known by the names of the men who first clearly formulated them.

Boyle's Law (1660)

Boyle's law states that if the temperature is constant the volume of a gas varies inversely proportionate to the pressure.

Boyle's law may be expressed as follows:

$$\frac{P_1}{P_2} = \frac{V_2}{V_1} \quad \text{or } P_1 \cdot V_1 = P_2 \cdot V_2 \quad \text{or } V_1 : P_2 = V_2 : P_1$$

The table below will aid in demonstrating the inverse relationship between volume and pressure as defined by Boyle's.

<u>Pressure</u>		<u>Volume</u>	
P_1	1 atm.	V_1	16 cc.
P_2	2 atm.	V_2	8 cc.
P_3	4 atm.	V_3	4 cc.
P_4	8 atm.	V_4	2 cc.
P_5	16 atm.	V_5	1 cc.

It is easy to see that as the pressure increases the volume decreases; and as the pressure decreases the volume increases (T being K).

Example of use of Boyle's Law

A volume of a gas measured at 740 mm. pressure is 200 ml.

(a) Calculate the volume of gas at standard pressure.

(b) Calculate the volume of gas at 610 mm. pressure.

(a) $P_1 = 740$

$P_2 = 760$

$V_1 = 200 \text{ ml.}$

$V_2 = \text{unknown}$

$$V_2 = 200 \times \frac{740}{760} = 194.7 \text{ ml.}$$

$$740 \times 200 = 760 \times V_2$$

$$V_2 = \frac{148000}{760} = 194.7 \text{ ml.}$$

Student exercise:

Solve part (b).

Charles' Law (1801)

Charles' law states that if the pressure remains constant the volume of a gas will vary directly as the absolute temperature.

Charles' law may be expressed as follows:

$$\frac{V_2}{V_1} = \frac{T_2}{T_1} \quad \text{or} \quad V_2 \cdot T_1 = V_1 \cdot T_2 \quad \text{or} \quad V_1 : T_1 = V_2 : T_2$$

Therefore, it can be seen that as the temperature increases the volume increases, and as the temperature decreases the volume decreases (P being K).

Example of use of Charles' Law

A volume of gas measured at 70°C. is 300 ml. Find its volume at:

(a) 0°C.

(b) 100°C.

A $T_1 = 70^\circ\text{C.}$

$T_2 = 0^\circ\text{C.}$

$V_1 = 300$

$V_2 = \text{Unknown}$

$$\frac{V_2}{V_1} = \frac{T_2}{T_1} \quad \text{or} \quad V_2 \cdot T_1 = V_1 \cdot T_2$$

$$\frac{V_2}{300} = \frac{273}{343} \quad V_2 \cdot 343 = 300 \cdot 273$$

$$V_2 = \frac{300 \times 273}{343} \quad V_2 = \frac{300 \times 273}{343}$$

$$V_2 = \frac{81900}{343} \quad V_2 = 238.8 \text{ ml.}$$

Student Exercise: Solve part (b)

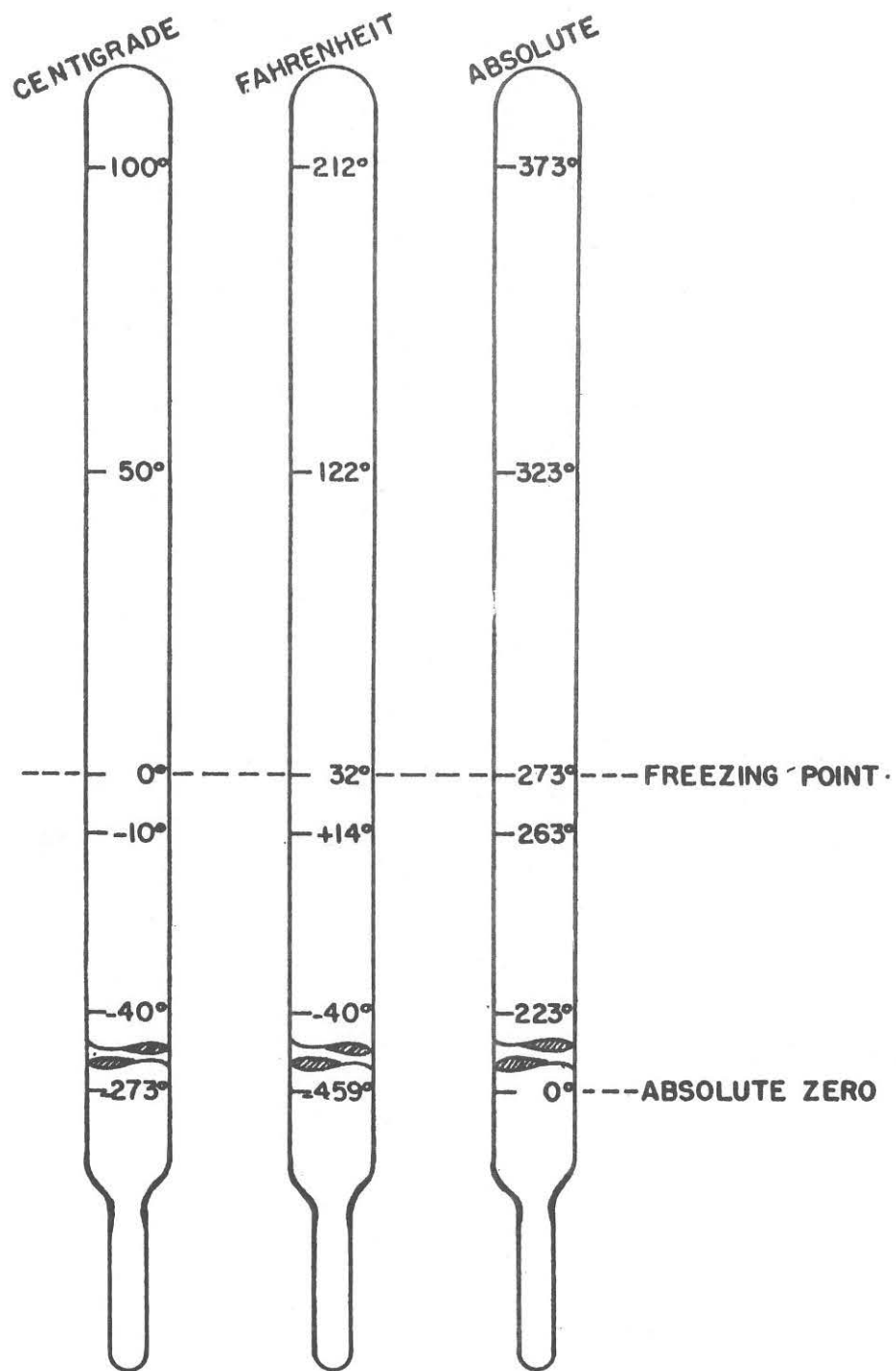


Figure 20. Comparison of thermometer scales.



Figure 26. Comparison of the three scales.

It has been demonstrated that all gases expand to the same extent when exposed to the same increase in temperature (P is K). It is further shown that a 1°C. rise in the temperature of a gas will expand the gas by 1/273 part of its volume at 0°C; a rise of 2°C. will increase the volume by 2/273 of its volume at 0°C; or a 273 ml. volume of a gas would become 274 ml. and 275 ml. respectively. Also a 1°C. fall in temperature of 273 ml. of gas will become 272 ml. or be reduced by 1/273 part of its volume at 0°C. For the purpose of gas calculations we use a "Absolute" or "Kelvin" scale of temperature at which 273°A = 0°C.

The value of 1°C. and 1°A is the same (Fig. 20, p. 62a) except that the freezing point on the absolute scale is 273°A and boiling point is 373°A as opposed to 0°C. to 100°C. on centigrade scale.

It is the absolute temperature that is always used when calculating to correct for temperature changes in the gasometric analysis.

It will be of value to the technician to know the general formulas for conversion from the Fahrenheit to the Centigrade scale and from the Absolute to the Centigrade as seen in Figure 20.

$$212^{\circ}\text{F} = 100^{\circ}\text{C} = 373^{\circ}\text{A}$$

Conversion of Fahrenheit to Centigrade

$$C = (F-32) \times 5/9 \text{ or } C = \frac{F-32}{1.8}$$

$$C = (212-32) \times 5/9 \quad C = \frac{212-32}{1.8}$$

$$C = 180 \times 5/9 = 100 \quad C = \frac{180}{1.8} = 100^{\circ}\text{C}.$$

Conversion of Centigrade to Fahrenheit

$$F = (100 \times 9/5) + 32$$

$$F = (100 \cdot 1.8) + 32$$

$$F = (20 \times 9) + 32$$

$$F = 180 + 32$$

$$F = 180 + 32 = 212^{\circ}$$

$$F = 212^{\circ}$$

Centigrade to Absolute (or Kelvin)

$$^{\circ}\text{C} + 273 = ^{\circ}\text{A}$$

If Boyle's and Charles' Laws are combined:

$$V \propto (1/P) \times T$$

and in general: $V = nR \times T/P$ or $PV = nRT$

where n represents the number of moles of gas present and R is the molar gas constant, the numerical value for which depends upon the units in which the pressure and volume and temperature are expressed. For pressure in atmospheres, volume in liters and temperature in degrees absolute, R is equal to 0.08205 liter-atmospheres per mole per degree.

Thus if we know the volume in liters, the pressure in atmospheres, and the temperature in absolute degrees, we can calculate the number of moles of gas present.

Dalton's Law

$$P_T = P_1 + P_2 + P_3 + P_4 + \dots + P_n$$

Dalton's Law states that the total pressure of a gas mixture is the sum of the pressures of the individual gases in the mixture.

Henry's Law

$$C_1 = kP$$

Henry's Law states that the solubility of a gas in a liquid is proportional to the pressure of the gas. Each gas has its own solubility coefficient k . Temperature increases will decrease the solubility of gases in liquids so k will vary with the temperature.

If the amount of a gas present is to be expressed in terms of a volume, the temperature and the pressure must be stated. The usual values chosen for a standard temperature and pressure are:

0°C. (273° absolute)

760 mm. Hg (mercury) (one atmosphere)

When it is necessary to convert a volume of gas at one temperature and pressure to the volume at another temperature and pressure the following formula is useful; the subscript "1" refers to one set of conditions and "2" to another.

$$\frac{P_1 V_1}{T_1} = \frac{P_2 V_2}{T_2}$$

Example: A gas measured at 20°C. and at 740 mm. Hg. pressure had a volume of 30.7 ml. What would be its volume measured under standard conditions?

$$\frac{740 \times 30.7}{20 \div 273} = \frac{760 \times V_2}{273}$$

$$V_2 = (740/760) \times (273/293) \times 30.7$$

$$= 0.974 \times 0.932 \times 30.7$$

$$= 27.8 \text{ ml.}$$

Note that the temperature correction factor and the pressure correction factor can be handled separately.

Water Vapor Pressure Correction:

When the volume of the gas is measured over water, part of the pressure (and part of the volume) is due to water vapor. The easiest way to handle this correction is to subtract the partial pressure of water vapor at the observed temperature from the total pressure to give the pressure due to the dry gas.

Temperature °C.	Vapor Pressure mm. Hg.	Temperature °C.	Vapor Pressure mm. Hg.
15	12.8	26	25.2
16	13.6	27	26.7
17	14.5	28	28.3
18	15.5	29	30.0
19	16.5	30	31.8
20	17.5	31	33.7
21	18.7	32	35.7
22	19.8	33	37.7
23	21.1	34	39.9
24	22.4	35	42.2
25	23.8		

Example: A gas sample is measured at 27°C., at 750 mm. Hg. pressure, and over water and its volume is found to be 34.2 ml. Calculate the volume of the dry gas at standard conditions.

At 27°C. the vapor pressure of water is 26.7 mm. Hg. (see table). Then using the formula:

$$\frac{P_1 V_1}{T_1} = \frac{P_2 V_2}{T_2}$$

$$\frac{(750 - 26.7) \times 34.2}{(27 \div 273)} = \frac{760 \times V_2}{273}$$

$$\begin{aligned} V_2 &= (723.3/760) \times (273/300) \times 34.2 \\ &= 0.952 \times 0.910 \times 34.2 \\ &= 29.6 \text{ ml.} \end{aligned}$$

Molar Gas Volume:

Equal volumes of all gases contain the same number of molecules. Thus each gas has a molar volume of 22.4 liters (at standard temperature and pressure). Thus we can calculate moles (and grams) from volumes and vice versa.

Example: Convert 29.6 ml. CO_2 (M. W. 44) at standard temperature and pressure to moles and also to grams.

$$\frac{29.6 \text{ ml.}}{22,400 \text{ ml.}} = \underline{0.00132 \text{ moles or } 1.32 \text{ mM}}$$

$$1.32 \times 44 = 58.2 \text{ mg. or } \underline{0.0582 \text{ g.}}$$

There are TWO GENERAL METHODS OF GAS ANALYSIS

(1) Volumetric - "volume measurement"

The pressure is "set" (usually at atmospheric pressure).

The volume is read (and also the temperature).

(2) Manometric - "pressure measurement"

The volume is set (usually at 2.0 or 0.5 ml.)

The pressure is read (usually in terms of mm. Hg.) (and the temperature is also read).

For further details of specific uses of gas analysis, see Carbon Dioxide, p. 129.

URINE COLLECTION AND PRESERVATION

In this work 24-hour samples of urine are usually used. Quantitative determinations of the constituents of random voidings are of no value or significance in the interpretation of metabolic processes. It is only when all urine produced by the kidneys over a known period of time is analyzed that conclusions can be drawn as to what the body is doing with various foodstuffs. The shortest period of time that is usually practicable for this purpose is an hour, but a twenty-four hour sample is usually preferable.

Collection of 24-hour urine specimen:

It is necessary to have a bottle of about two liters capacity, with a stopper or cover. The bottle should be clean. In it is placed 1 to 2 ml. of toluene to act as a preservative. (In some cases the use of toluene must be avoided).

At a convenient time in the morning (say 7 o'clock) the bladder is emptied and the specimen is discarded. This voiding represents urine produced by the kidneys before the time of collection. Thereafter all urine voided up to and including the 7 A.M. voiding (or the time corresponding to that of the discarded specimen of the previous morning) is placed in the bottle, which should be kept in a cool place. Analyses should be carried out as soon as possible after collection of the specimen.

If it is not possible to collect the entire 24-hour specimen in a single container, all portions of it should be poured into a single container and mixed well to make it homogeneous before any sample is removed. Thereafter the specimen should be stirred up well each time a sample is removed. Otherwise, more or less of the sediment will be taken out than should go with the removed portion, the composition of the removed portion and of the remaining portion being changed thereby.

Measurement of volume:

Since the amount of a constituent in the whole specimen is found by calculation from the amount determined in an aliquot, it is necessary to know the exact volume of the 24-hour specimen. Hence the volume should be carefully measured before any other determination is made. Sometimes it is necessary to determine the separate volumes of the urine excreted in the first 12 hours and that excreted during the second 12 hours. Volume measurements can be carried out during collection of the specimen, but none of the specimen(s) should be discarded. Specimens for routine urine analysis with no quantitative determinations may be random, although it is much preferable to obtain morning specimens for this purpose.

Preservatives:

1. Toluene is a very acceptable preservative for urine for general use.
2. Formaldehyde or oxalic acid may be used in special cases.
3. Sodium carbonate 5 g. is used in addition to petroleum ether or toluene for the preservation of the biliary and porphyrin pigments.

4. A few ml. of glacial acetic acid may be used in cases in which maintenance of an acid pH is required such as Addis Counts and Calcium in urine.

5. The best method is storage in a refrigerator but this is not always practicable.

Changes during storage of urine:

1. Bacterial action. Since urine is usually not collected aseptically, it is not sterile. However, if clean containers are used, little change will be noted in a 24-hour collection period especially if it is kept cool and stoppered during the collection period.

Bacterial action on urea:

This results in the formation of ammonia, carbon dioxide, and a change in pH. Ammonia (and its nitrogen) may be lost to the air. If the urine becomes alkaline, calcium and magnesium phosphate salts will precipitate.

Bacterial action on glucose:

In a specimen containing glucose, the above actions may be accelerated and the glucose may disappear due to its utilization by the bacteria.

2. Uric acid and urates may precipitate on allowing the urine to stand, especially in the refrigerator. Before analysis, all sediments must be well suspended and a sample taken of the well mixed specimen. Other changes may occur, some of which are mentioned under the particular method when necessary.

FECES COLLECTION

For qualitative tests, such as for occult blood, only a portion of the stool collection need be sent to the laboratory. However, it is important that this portion be carefully selected, preferably by the physician ordering the test.

For quantitative tests, and especially for balance studies, complete collection of all stools passed is necessary. The stool should be collected in such a way that urine does not become mixed with the sample. In most cases, it is possible by inspection to detect urinary admixture. Considerable amounts of chloride also indicate urine contamination since feces contain little chloride ion.

Marking of feces:

In balance studies it is sometimes desired to collect all feces formed during a given period of time, say 3-4 days.

A neutral substance such as charcoal, carmine, gentian violet in amounts of 5 grains (300 milligrams) may be given in a gelatin capsule with the first meal of a given experimental period and again with the first meal after the experimental period has ended. Thus the first feces associated with the test period will be that first marked with the dye. The first feces after all test period samples have been collected will be marked with dye and should be discarded. Save the first marked specimen and all stools thereafter up to, but not including, the next marked specimen which should be discarded.

Sampling of Feces:

Since the constitution of feces is variable the problem of sampling the total collection is critical.

1. Dry mixing and sampling - the entire sample is dried, ground, and then well mixed. A properly withdrawn aliquot is then analyzed.

2. Wet mixing and sampling - this procedure uses a Waring blender to produce a homogeneous pipettable suspension.

Procedure:

1. The sample is collected into a large jar with a wide mouth, up to three days. The sample is always kept in a refrigerator.

2. At the end of that time, approximately 1500 ml. of water is added, and the lid of the jar stoppered tightly.

3. Add 1 ml. of caprylic alcohol to prevent foam, and shake vigorously.

4. Pour portions gradually into a Waring Blender and homogenize completely.

5. Add all the homogenized portions to a 2000 ml. graduated cylinder and dilute exactly to 2000 ml.

6. Pour all into a large bottle or flask, mix by shaking and pour aliquots into two 250 ml. brown bottles. Stopper well.

7. Deep freeze until used for analysis.

8. For individual analyses, see the appropriate determination.

9. For almost all kinds of analysis, aliquots may be measured accurately from a Yale BD Lok-Syringe, which delivers accurately to 0.1 ml. if properly handled.

Calculations:

If the collection period was three days, for any given constituent:

$$\text{g/day} = (\text{g/ml.}) \frac{2000}{3}$$

Precautions:

1. Fill bottles only 2/3 full, as in freezing the expansion will cause the bottles to crack if full.

2. Steps 4, 5 and 6 are critical. Unless the specimen is homogenized well enough to be pipetted accurately, results will be unreliable. No discrete particles must remain in the final mixture.

Preservation of feces:

Specimens should be analyzed as soon as collected to avoid the necessity of adding preservatives.

If feces must be preserved it is best to treat them as indicated under wet mixing above.

COLLECTION OF BLOOD SPECIMENS

The clinical analysis of blood (as well as of urine and other biological materials) really starts with the patient. In order to obtain as reproducible results as possible, most blood samples are collected from patients in a "post-prandial" state. This is the time at about 12-14 hours after food has been taken, usually in the morning before breakfast. This procedure minimizes the effects due to digestion and absorption of foodstuffs on the one hand, and the effects of "starvation" (i.e., mobilization of tissue fat and carbohydrate) on the other. In some analyses it is also important to limit the motor activity of the patient (e.g., by keeping him in bed) before the sample is taken.

There are a number of routes by which blood samples may be taken. They will be noted here, together with a brief outline of the essential points of the procedure.

I. Capillary Collection:

Capillary blood is most frequently obtained from a finger or thumb, ear lobe or heel (in infants). The site is first cleaned with alcohol or acetone, the solvent is allowed to dry and the site is then pricked quickly with a sterile needle. A freer flow of blood is obtained if the area is warmed for 10-15 minutes before a collection, and if this is done, the characteristics of capillary blood closely approximate those of arterial blood, especially when taken from finger or thumb. The prick should be deep enough to insure sufficient flow for the collection.

This procedure is usually used when it is desired to collect 0.1 to 0.2 ml. of blood although up to 2 ml. can sometimes be obtained by this method.

The blood is usually collected directly into pipets calibrated "to contain." The bore is quite small so that the blood runs into them without aspiration if they are kept horizontal. This is true only if they are scrupulously clean.

The blood is allowed to flow into the pipet until it is filled to just above the calibration mark, the upper end is closed off with the top of the forefinger. After adjusting exactly to the mark by tipping the end of the pipet to a filter paper, or to the finger, the pipet is wiped clean and the contained blood is allowed to run into a tube containing water or other diluting solution. By alternately blowing and aspirating, the blood is washed from the pipet by the solution. Various types of solution may be used depending upon the analysis to be made. See deproteinization, p. 76.

II. Venous Collection:

Since usually more than one determination is to be carried out, venous collection is preferable. The blood is usually withdrawn from a vein in the antecubital space of the arm. The syringe used should be large enough to collect the entire specimen. In most cases, the tourniquet used to demonstrate the vein should be removed before the blood sample is collected since stasis of the blood alters some of the clinically significant blood values. For details and illustrations of the technic of venipuncture see The Handbook of the Medical Corps, U. S. Navy and also the Serology Manual.

After the sample has been obtained and the needle removed from the vein, pressure

should be maintained on the site to prevent peri-vascular hemorrhage. The blood in the syringe is then promptly transferred to plain tubes, or to tubes containing anti-coagulant (see below).

III. Arterial collection:

Collection directly from arteries is rarely made. It may be taken for blood-gas studies. It is usually withdrawn from the radial or brachial or the femoral artery and usually under local anesthesia.

Prevention of Hemolysis:

In many determinations it is preferable to use serum. Certain determinations cannot be run if there is even a small amount of hemolysis. It is worthwhile, therefore, to note the steps that are necessary to prevent hemolysis:

1. The syringe and needle used must be dry.
2. The needle used must be of a sufficiently large bore to allow the blood to run through it without being forced to too high a velocity. A 20-gauge needle is satisfactory.
3. Blood should be allowed to flow into the syringe; one should not pull back hard on the plunger.
4. The needle should be removed before the blood is transferred into the clean, dry test tube. The blood should be allowed to run gently down the wall of the tube and should not be squirted into it.
5. The tube should be allowed to stand quietly for 10 to 15 minutes without being agitated. It should not be allowed to roll around on the bottom of a tray.

The same steps can be followed in obtaining oxalated blood except that it is necessary to stopper the tube and invert gently a number of times as soon as the blood is placed in it, to dissolve and mix the oxalate.

Centrifugation:

When centrifugation is to be carried out the following principles must be observed to avoid damage to the centrifuge or to the operator.

1. The principle of "balance" must be observed. Tubes and cups of equal weight, shape, and size should be placed in opposing positions in the centrifuge head.
2. Tubes should be buffered by rubber cushions in the brass cups, and additional cushioning may be obtained by using water in the brass cups.
3. The glass tubes used should be inspected to insure that they have no cracks or chips. Heavy stoppers, (either rubber or glass) should not be used. Corks are preferable in most cases.
4. When these precautions have been observed, the lid of the centrifuge is closed, and the machine is slowly speeded up until the desired speed is attained. After the required length of time, the power is cut off and the centrifuge allowed to come to rest by itself. Rapid slowing by a brake can stir up precipitated material.

Choice of sample:

When carrying out blood analysis we may choose to use whole blood, serum, or plasma. Serum is preferred to whole blood or plasma whenever its use is practical. There are a few general principles to be considered in the choice of the sample to be analyzed.

1. If the substance analyzed for is evenly distributed between red blood cells and plasma (or serum) it does not matter which is used. If a capillary collection is done, whole blood may be more convenient.
2. Some substances present in erythrocytes may adversely affect the accuracy of the determination by interfering in the reaction. In such cases it is better to use serum or plasma.
3. In some cases the clinically significant variation in concentration occurs only in the serum or plasma, for example in serum chloride, which varies much more with disease states than does whole blood chloride since the red blood cell chloride concentration is relatively stable.
4. If there are great differences in concentration between rbc and plasma, variations in hematocrit will greatly affect the results.
5. It is easier to avoid hemolysis in the production of serum, but when the separation and analysis must be made quickly plasma is usually chosen.

Preparation of blood for analysis:

After the blood has been drawn from the patient, it is treated in various ways to obtain suitable samples for analysis.

1. Serum:

Serum should be used wherever it is practicable. To obtain serum the needle is removed and the blood in the syringe is transferred without undue trauma to a clean dry test tube which can be centrifuged. The tube is allowed to stand quietly without agitation for 15-30 minutes. The clot is then loosened from the wall of the test tube with a glass rod or applicator stick and the tube is centrifuged for about 5 minutes at about 1500-2000 rpm, stoppered with a light rubber stopper or a cork. The serum is removed by the use of a rubber bulb fitted to a glass tubing, the end of which is drawn out to a coarse capillary. If any rbc are inadvertently included in removing the supernatant, the serum should be recentrifuged before sampling.

2. Plasma:

If plasma is to be obtained an anticoagulant must be promptly mixed with the withdrawn blood to prevent clotting. Most of these act by combining chemically with the Ca^{++} of the blood to prevent its participation in the clotting mechanism. Oxalate and fluoride act by forming insoluble calcium oxalate and calcium fluoride. Citrate and EDTA (ethylene-diamine-tetracetic acid) act by forming un-ionized calcium salt complexes; heparin acts (a) in the first stage of coagulation to prevent the production of plasma thromboplastin, (b) in the second stage to inhibit the formation of thrombin from prothrombin (and apparently as an anti-accelerin) and (c) in the third stage to inhibit the action of thrombin on fibrinogen. Heparin acting alone is not an anticoagulant. It acts in conjunction with certain albumin fractions of plasma. Due to lack of this albumin factor rabbit blood will clot in the presence of large amounts of heparin.

Anticoagulants:

1. The anticoagulant most commonly used in the clinical chemistry laboratory is potassium oxalate, $K_2C_2O_4 \cdot H_2O$. This salt can be made up in a 30% solution by dissolving 30 g. in enough water to make 100 ml. of solution. One-tenth milliliter of this solution contains 30 mg., an amount sufficient to prevent the coagulation of 15 ml. of properly drawn blood. If smaller samples of blood are anticipated, correspondingly lesser amounts of the anticoagulant solution should be employed (2 mg. of $K_2C_2O_4 \cdot H_2O$ per milliliter of blood). The proper amount of solution is introduced into the bottom of a clean, dry, test tube which is then rolled on its side on a hot plate until water has evaporated, leaving the oxalate as a loose salt deposit scattered over the walls of the tube. A high temperature should be avoided in order to keep from converting the oxalate to carbonate. Such tubes can be prepared in quantities, stoppered, and stored indefinitely.

2. Mixed oxalate (for hematology). The above salt makes the plasma somewhat hypertonic and causes some water to be drawn out of the cells with consequent dilution of plasma constituents and shrinkage of the cells. If it is desired to prevent such shift of water and if the presence of ammonium ions does not interfere with subsequent uses, a solution of ammonium oxalate, $(NH_4)_2C_2O_4 \cdot H_2O$, and potassium oxalate can be made as follows: Dissolve 6 g. of $(NH_4)_2C_2O_4 \cdot H_2O$ and 4 g. of $K_2C_2O_4 \cdot H_2O$ in water, dilute up to a volume of 100 ml., and mix. One-tenth ml. of this solution contains enough oxalate for 5 ml. of blood.

3. Fluoride (in the form of sodium fluoride), NaF acts as an anticoagulant but is required in much larger amounts (100 mg./10 ml. of blood) than is necessary for oxalates. It is commonly used in combination with thymol and oxalate whenever blood must be preserved for later analysis. Fluoride inhibits the action of certain enzymes (such as urease) and this effect must be kept in mind in certain analyses. The following mixture of reagent grade chemicals well mixed is often used in the amount of 5 mg./ml. of blood.

Sodium fluoride, powder (NaF)	10 parts
Thymol, powder	1 part
Potassium oxalate, powder	3 parts
$(K_2C_2O_4 \cdot H_2O)$	

4. Citrate. Sodium and potassium citrates can be used as efficient anticoagulants, especially in the collection of blood for transfusion. For analytical purposes, citrate has the disadvantage of causing marked shifts of volume and acidity between erythrocytes and plasma. About 60 mg. sodium citrate ($Na_3C_6H_5O_7 \cdot 2$ (or 5) H_2O) should be used for each 10 ml. of blood. For prothrombin times, some workers prefer the use of 3.8% sodium citrate instead of 2% potassium oxalate.

5. EDTA - disodium salt. This is soluble to about 10%. It should be neutralized to pH 7.4 before use by the addition of NaOH. The use of 10 mg./10 ml. of blood suffices to prevent coagulation. For practical use 2 drops of the 10% solution may be used for each 10 ml. of blood. It is preferable to dry this solution in the collecting tube but if it is not dried, the error by dilution will be about 1% (0.1 ml. in a total volume of 10 ml.) The use of EDTA has some advantages in the determination of sedimentation rates, hematocrit, and platelet counting. The potassium salt is fifteen times as

soluble as the sodium salt and thus may be more convenient to use.

References to EDTA use:

Am. J. Clin Path. 23, 613 (1953)

" " " " 25, 1090 (1955)

USAF Med. J. 4, 1556 (1953)

6. Heparin. This substance is a naturally occurring substance found in liver and lung and is extremely effective in preventing the coagulation of blood. The very pure preparations require only 1 mg./100 ml. The usual preparations are effective when used in the proportion of 0.5 mg./10 ml. of blood. Preparations are available as the sodium salt and as the calcium salt. Some preparations have significant amounts of phosphate as well. This inorganic ion content should be considered when heparin is used as an anticoagulant.

Precautions:

1. With all of these anti-coagulants, if plasma is to be used, it should preferably be separated from the rbc as quickly as possible.

2. If whole blood is to be used, it is extremely important to thoroughly mix the sample of blood before a sample is withdrawn. If this is not done, any constituent with unequal distribution (by volume) in erythrocyte and plasma will be estimated with an original "built-in" error. This error, due to inadequate mixing, is very common in most clinical chemistry laboratories. It can be eliminated only by unending vigilance on the part of the director and the individual laboratory technicians.

Changes in stored blood:

Certain changes in the chemical constitution of blood, some of which are listed below, occur when blood is stored. In general, it is best to analyze samples as soon as possible after collection. If serum or plasma is to be used, prompt separation from the cell mass is recommended.

Some of the possible changes are:

1. Loss of carbon dioxide: this is more rapid when the exposure to the air is increased. When this occurs, the cells are involved by way of the chloride shift (see p. 34). To minimize this loss of carbon dioxide, blood may be collected without contact with air in "Vacutainer" tubes (see p. 75), under oil or under mercury, or merely kept in a capped syringe with suitable anticoagulant until it is analyzed.

2. Glucose is metabolized: the red blood cells utilize glucose and part at least is converted to lactic acid. This results in a low blood sugar and also in pH changes in the blood.

3. Plasma inorganic phosphate may increase due to hydrolysis of sugar phosphate esters in the rbc and their transfer to the plasma. Prompt separation of plasma or serum avoids this.

4. Ammonia may be formed by bacterial action on the urea of blood, and this process is quite rapid in contaminated specimens. This formation of ammonia interferes in the cobalt-nitrite chemical method for potassium.

5. Some substances diffuse from the rbc to plasma. Potassium will diffuse (a) from plasma to rbc at first, (b) later from rbc to plasma. If whole blood is kept cold,

the cell potassium will diffuse to the plasma. Prompt separation of serum and cells prevents this type of error.

After separation, the samples of serum or plasma may be stored in the refrigerator or frozen. Details of the effects of various types of storage on the individual determinations will be mentioned specifically (when they are known) under each method.

"Vacutainer" Tubes:

It is possible to obtain special evacuated tubes, which can be prepared containing anti-coagulant, from several commercial sources. These make it possible to collect venous blood without exposure to air and thus without loss of carbon dioxide.

PROTEIN-FREE FILTRATES

Introduction:

In most of the determinations to follow, the removal of protein from the solution is the first step in the analysis. Proteins remain in solution because the forces tending to separate the individual protein molecules are greater than the forces tending to bring them together. These separating forces are:

1. Electrical charge on the protein. This is present whenever the protein is away from its isoelectric point, i.e., when it is ionized.

2. Hydration. Since most proteins contain many polar (hydrophilic) groups, a shell of water forms around the protein molecule.

Any procedure tending to decrease the magnitude of these two separating forces will tend to precipitate the protein.

Some general procedures used to precipitate proteins are as follows:

1. Adjustment of pH to the iso-electric point. Some proteins precipitate when brought to their iso-electric point. For example: the casein of milk will precipitate when the milk is "soured" or "brought to the iso-electric pH of casein." The iso-electric pH (or point) is the pH at which the number of negative charges on the protein particle equals the number of positive charges; i.e., there is a zero net charge.

2. Alteration of hydration by the use of (a) strong salt solutions, (b) non-aqueous solvents (such as alcohol) at low temperatures and (c) evaporation of water at low temperatures (lyophilization) all serve to precipitate proteins and usually result in little or no alteration in the structure or natural state of the protein; i.e., little or no "denaturation."

3. Denaturation of a protein produces a change (usually a decrease) in solubility. This denaturation may be brought about by (a) heat, (b) non-aqueous solvents at room or elevated temperatures, (c) strong acids or alkalies, and (d) other types of reagents or physical procedures.

4. When proteins are charged (ionized) they will combine with other ions to produce insoluble compounds.

- (a) When they have a positive charge, i.e., they are cations (existing as protein⁺) they will combine with "heavy anions" (called alkaloidal reagents) such as picrate, ferrocyanide, tungstate, etc.; to give less ionization.

- (b) When they have a negative charge, i.e., they are anions (protein⁻) they will combine with heavy metal ions or other heavy cations such as mercuric, lead, zinc, etc., to give decreased ionization.

With each of these methods using heavy cations or heavy anions, there is a more or less complete denaturation of the protein when the precipitation occurs at room temperature or above. If precipitation with heavy metals is carried out at low temperatures (0°C.) the protein may be recovered in essentially its native state.

There have been many different procedures used for the preparation of protein-free filtrates. The most commonly used one is that involving tungstic acid. Unless otherwise noted in the specific method, a protein-free filtrate refers to a filtrate prepared as noted in the tungstic acid methods below.

METHOD I

Reference:

Folin, O., and Wu, H., A system of blood analysis, J. Biol. Chem., 38, 81 (1919).

Reagents:

1. 10% sodium tungstate. Dissolve 100 g. reagent grade, carbonate free, sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) in water and dilute to 1 liter, mix. This solution is stable indefinitely.

2. $\frac{2}{3}$ N H_2SO_4 . Weigh out 35 g. of concentrated sulfuric acid in a small tared beaker, dilute to 1 liter with water and mix. Check by titration against standard alkali and adjust if necessary.

Procedure:

To 1 volume of blood in an Erlenmeyer flask, add exactly 7 volumes of water, mix. Add 1 volume of 10% sodium tungstate and mix. Add slowly and with shaking 1 volume of $\frac{2}{3}$ N H_2SO_4 . Stopper the flask securely and shake hard. Only a few bubbles should form and a hard metallic splash should be heard. Let stand 3 minutes. Pour slowly on to a dry filter and collect the filtrate.

METHOD II A.

Reference:

Haden Modification of Folin Wu protein-free filtrates: J. Biol. Chem., 56, 469 (1923)

Principle:

In this modification the blood is laked and proteins given a positive charge by the acid; i.e., they become cations. Then, addition of the tungstate results in the formation of protein-tungstates. This procedure employs fewer solutions and gives more filtrate than Method I.

Reagents:

1. 10% sodium tungstate, as in Method I.

2. N/12 H_2SO_4 . Add 2.5 ml. of concentrated H_2SO_4 to 1 liter of distilled water. Mix well and check by titration against 0.1 N NaOH so that 20 ml. of N/12 H_2SO_4 requires 16.7 ml. of 0.1 N NaOH for neutralization.

Procedure:

1. In a 250 ml. flask place 40 ml. of N/12 H_2SO_4 .

2. Add slowly, 5.0 ml. of blood, mixing well. Allow to stand 1 minute.

3. Add 5.0 ml. sodium tungstate 10%, stopper, shake vigorously; let stand 3 minutes.

4. Pour slowly on to a dry filter.

In these two procedures the protein precipitate is or becomes a chocolate brown and the filtrate should be absolutely clear and colorless. It contains all blood constituents except protein, diluted 10 times.

METHOD II B.

Principle:

Since it is desirable to bind all the tungstic acid, when serum or plasma is used, a modification must be made since less protein is present. Some workers have recommended 50% of the amounts used for whole blood, but we have found, along with many others, that 70% gives somewhat better results.

Reagents:

1. Dilute 7 volumes of $N/12$ H_2SO_4 up to 10 volumes with water. This dilution gives 0.058 N H_2SO_4 .

2. Sodium tungstate 7%. Dilute 7 volumes of $Na_2WO_4 \cdot 2H_2O$ (10%) up to 10 volumes with water.

Procedure:

To eight volumes of 0.058 N H_2SO_4 add 1 volume of serum or plasma. Mix. Add 1 volume of 7% sodium tungstate and shake vigorously. Filter through filter paper, or centrifuge.

METHOD III.

Reference:

Van Slyke, D. D. and Hawkins, D., Pre-mixed Tungstic Acid, J. Biol. Chem., **79**, 739 (1928).

Principle:

The sodium tungstate and the sulfuric acid are mixed before the blood, serum or plasma, or cerebro-spinal fluid is added. The tungstic acid produced is unstable and should be freshly prepared weekly. This procedure is especially recommended when a large number of filtrates are made daily.

Reagents:

Mix: 1 volume of $2/3$ N H_2SO_4
7 volumes of distilled H_2O
1 volume 10% Na_2WO_4 (sodium tungstate)

or

8 volumes $N/12$ H_2SO_4
1 volume 10% Na_2WO_4

Prepare fresh weekly. Small amounts of precipitated tungstic acid do not interfere.

Procedure:

A. Whole Blood: To 9 volume of pre-mixed tungstic acid reagent, add 1 volume of whole blood. Shake and filter.

B. Serum and Plasma: To a mixture of 5 volumes of tungstic acid reagent and 4 volumes of distilled water add 1 volume of serum (or plasma) dropwise with continual shaking. Shake vigorously and allow to stand for 10 minutes. Filter.

C. Cerebro-spinal fluid: To a mixture of 2 volumes of tungstic acid reagent and 2 volumes of distilled water add 1 volume of spinal fluid. This obviously results in a 1-5 dilution instead of the usual 1-10, and this should be taken into consideration in the calculation of results.

METHOD IV

Zinc sulfate-barium hydroxide

Reference:

Somogyi, M., J. Biol. Chem., 160, 69 (1945).

Principle:

The reaction between zinc sulfate and barium hydroxide is complex, but in essence, results in the precipitation of two insoluble products, zinc hydroxide and barium sulfate, which bind and occlude the protein, removing it from solution. This procedure has the advantage of adding no extraneous ions to the solution.

Reagents:

1. Zinc sulfate reagent 5%. Dissolve 50.0 g. of $\text{ZnSO}_4 \cdot 6\text{H}_2\text{O}$ (or $7\text{H}_2\text{O}$) in water. dilute up to 1000 ml. and mix.

2. Barium hydroxide 0.30 N. Dissolve 50 g. of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ and dilute up to 1000 ml. of water. This solution must be balanced against the zinc sulfate as follows:

Pipet into a flask 25.00 ml. of the zinc sulfate solution. Add phenolphthalein indicator and titrate dropwise with the $\text{Ba}(\text{OH})_2$ solution, using vigorous stirring, to a definite permanent pink color. Adjust the concentration of the barium hydroxide or the zinc sulfate. Store the alkaline solution with protection from carbon dioxide of the air.

3. Dilute reagents. Add four parts of water to one part of the reagent.

Procedure A. (For a 1-10 filtrate)

1. Add to 5.0 ml. distilled water, 1.0 ml. of sample (whole blood, serum or plasma). To this add 2.0 ml. of 0.3 N $\text{Ba}(\text{OH})_2$. Mix and allow to stand 3 to 5 minutes.

2. Add 2.0 ml. 5% ZnSO_4 and shake vigorously.

3. Filter through a dry filter paper into a dry container (or centrifuge) to obtain a clear filtrate.

Procedure B. (For a 1-21 filtrate)

1. Pipet 10 ml. of dilute barium hydroxide into a small dry flask and add 1.0 ml. of blood, serum or plasma. Mix well and allow to stand 3-5 minutes.
2. Add 10.00 ml. of dilute zinc sulfate reagent, stopper and shake.
3. Filter through dry filter paper into a dry container or centrifuge. Notice that in this case the total volume is 21 ml. and the dilution is 1-21.

Note 1.

The use of barium-zinc deproteinization has the advantage of somewhat greater speed and somewhat larger volumes of filtrate can be obtained. It removes some of the nitrogenous constituents contributing to the non-glucose reducing substances and therefore blood glucose values closer to the actual glucose concentration are obtained. It also results in lower normal non-protein nitrogen values and clearly the filtrates cannot be used for the determination of uric acid or creatinine since these do not appear in the filtrate.

Note 2.

Other volumes and concentrations can be used provided the same proportion of reagents is used (2 ml. each of 5% ZnSO_4 and 0.3 N $\text{Ba}(\text{OH})_2$ for each ml. of blood). By this method a clear filtrate may be obtained regardless of how small an amount of protein may be in the biological fluid. This is not true of the tungstic acid filtrate. The mixture can also be centrifuged and an aliquot removed by pipet or by decantation.

Note 3.

Sodium hydroxide may be substituted for $\text{Ba}(\text{OH})_2$ of the same normality, when whole blood is used, but some protein is left in solution when serum or plasma is used, unless heat is also applied.

Other methods of precipitation:

The methods mentioned above, are those upon whose use is based most of our basic clinical values, together with the pathological variations characteristic in diseases states. In some cases, the use of tungstic acid filtrates has been found unsatisfactory and other methods have been resorted to. Examples of these will in most cases be found in the manual associated with those methods. These are reagents such as zinc and copper hydroxides, trichloroacetic acid, picric acid, etc. In addition there are numerous modifications of the tungstic acid and the metallic hydroxide procedures, each with some particular advantage. Those outlined above will suffice for most purposes.

METHOD V

Micro-filtrates:

In some cases, the amount of sample available may be limited, making it necessary to reduce the volume of the reagents proportionally. Whenever a "to contain" pipet is used, it should be remembered that a "rinse-out" technique is required.

Procedure A. Micro-modification of Method II A

Into a test tube, which can be centrifuged, introduce 1.60 ml. $N/12 H_2SO_4$. From a finger tip (or heel or ear lobe) prick, collect 0.20 ml. of blood in a "to contain" pipet. Rinse back and forth in the 1.60 ml. $N/12 H_2SO_4$ at least eight times and then add 0.20 ml. 10% sodium tungstate. Mix well. (The acid and tungstate may be mixed prior to addition of the blood.) Centrifuge and pipet 1.0 ml. of the supernatant for analysis.

Procedure B. Micro-modification of Method III A.

Into a test tube, which can be centrifuged, add 1.8 ml. pre-mixed tungstic acid reagent. From a 0.2 ml. "to contain" capillary pipet, introduce the sample of blood, rinsing back and forth at least 8 times. Centrifuge and pipet an aliquot for analysis.

Procedure C. Micro-modification of Method IV B.

To 2.0 ml. of 0.06 $N Ba(OH)_2$ or (NaOH) add, using a 0.2 ml. "to contain" pipet, 0.2 ml. blood, rinsing as described above. Add 2.0 ml. 1% $ZnSO_4$, mix well, centrifuge and use the supernatant as a 1-21 diluted blood filtrate.

Procedure A. Micro-modification of Method II A.

Into a test tube, which can be centrifuged, introduce 1.00 ml. $N/12\ H_2SO_4$. From a finger tip (or heel or ear lobe) prick, collect 0.20 ml. of blood in a "no contain" pipet. Rinse back and forth in the 1.00 ml. $N/12\ H_2SO_4$ at least eight times and then add 0.20 ml. 10% sodium tungstate. Mix well. (The acid and tungstate may be rinsed prior to addition of the blood.) Centrifuge and pipet 1.0 ml. of the supernatant for analysis.

Procedure B. Micro-modification of Method III A.

Into a test tube, which can be centrifuged, add 1.0 ml. pre-washed tungstic acid reagent. From a 0.2 ml. "no contain" capillary pipet, introduce the sample of blood, rinsing back and forth at least 8 times. Centrifuge and pipet an aliquot for analysis.

Procedure C. Micro-modification of Method IV B.

To 2.0 ml. of 0.06 $N\ H_2SO_4$ or $H_2C_2O_4$ add, using a 0.2 ml. "no contain" pipet, 0.2 ml. blood, rinsing as described above. Add 0.2 ml. 1% Na_2SO_4 , mix well, centrifuge and use the supernatant as a 1-21 diluted blood dilution.

ACETONE (and ACETONE BODIES) in SERUM

References:

- (1) Dumm, R. M., and Shipley, R. A., The Simple Estimation of Blood Ketones in Diabetic Acidosis. J. Lab. Clin. Med. 31, 1162 (1946).
- (2) Greenberg, L. A., and Lester, D., A Micro-Method for the determination of Acetone and Acetone Bodies. J. Biol. Chem. 154, 177 (1944)
- (3) Shipley, R. A., and Long, C. N. H., Studies on the Ketogenic Activity of the Anterior Pituitary. Biochem. J. 32, 2242 (1938).

Principle:

Sodium nitroferricyanide (sodium nitroprusside) in the presence of weak alkali (ammonia) will give a purple color with aceto-acetic acid and with acetone. B-hydroxybutyric acid does not give a positive test. The test applied to acetoacetic acid is five to ten times more sensitive than when applied to acetone.

Reagents:

Acetone Test Powder

Grind separately (if not a fine powder) the following ingredients:

Sodium nitroferricyanide	1 gram
Ammonium sulfate (dry)	20 grams
Sodium carbonate (anhydrous)	20 grams.

The three ingredients are mixed, without grinding, in a screw-capped bottle. The mixture must be kept dry at all times and under these conditions is stable for three months or longer.

Procedure:

1. For each test, place a pinch of the mixed powder, 5 millimeters in diameter, on a circle of white filter paper. Add one drop of serum without stirring. A positive test is indicated by a red to purple color.

2. If a positive test is obtained by undiluted serum, make successive dilutions by adding water to the sample, testing each dilution as above.

This procedure may be applied to urine and to spinal fluid.

Calculations:

The minimal concentration detectable under these conditions is 10 mg. acetone per 100 ml. serum. Thus the dilution factor multiplied by 10 gives the approximate concentration of acetone bodies (expressed as acetone) present in the original serum.

Example:

A serum from a diabetic in coma was successively diluted and tested. The dilution of (1 / 5) gave a positive test and that of (1 / 6) gave a negative test.

$$6(\text{dilution factor}) \times 10 = 60 \text{ mg. \% acetone}$$

Standardization:

In order to check the reliability of the test, a known acetone solution of about 50 mg. % acetone may be prepared as follows: To 100 ml. of distilled water add 2 drops pure acetone - mix well. Use as serum in the procedure outlined above.

Interpretation:

The normal serum contains 1 - 6 mg. of acetone bodies (as acetone). In diabetes elevations up to 300 - 400 mg. per 100 ml. may be seen. In normal urines up to 50 mg. per day are seen while in diabetes 10 to 50 grams per liter may be found - over half in the form of B-hydroxybutyric acid (which does not react in this test).

This test is valuable in distinguishing between true diabetic acidosis in which the serum acetone exceeds 50 mg. % and surgical indications in a diabetic, e.g., acute abdomen with vomiting, etc., in which the blood level rarely exceeds 20 mg. %.

ADRENAL INSUFFICIENCY TEST

(Kepler-Power Water Test)

References:

- (1) Robinson, F. J., Power, M. H., and Kepler, E. J.: Proc. Mayo Clinic **16**, 577 (1941)
- (2) Levy, M. S., Power, M. H., and Kepler, E. J.: J. Clin. Endocrinol. **6**, 607, (1946)
- (3) Cutler, H. H., Power, M. H., Wilder, R. M.: J.A.M.A. **111**, 117 (1938)

Principle:

Patients having Addison's disease show

- (1) a decreased ability to rapidly excrete water after increased intake
- (2) excessive amounts of NaCl are excreted and there is a tendency to retain urea.

Procedure 1: (Based on volume of urine)

The Water Test - On the day before the test the patient eats three ordinary meals but omits extra salt. He is requested not to eat or drink anything after 6 o'clock in the evening. Until this time he may drink water as desired. At 10:30 P.M. he is requested to empty his bladder and discard the urine. All urine which is voided from then on until and including 7:30 A.M. is collected. The volume of this urine is measured and saved for chemical analysis if this should be necessary later. Breakfast is omitted. The patient is asked to void again at 8:30 A.M. and immediately thereafter he is given 20 ml. of water per kilogram of body weight (9 ml. per pound). He is asked to drink this within the next forty-five minutes. At 9:30, 10:30, 11:30 A.M. and at 12:30 P.M. he is requested to empty his bladder. In order to eliminate the effects of exercise and posture on urinary excretion, he is kept at rest in bed except when up to void. Each specimen is kept in a separate container. The volume of the largest one of these four specimens is measured.

Under these conditions some patients having Addison's disease will excrete so little urine that they are unable to void more than once or twice during the entire morning. In such instances the amount of urine excreted per hour may be calculated; frequently however, such calculations are unnecessary because of the very low urinary output throughout the entire morning.

Inferences that may be drawn from Procedure 1: These are as follows:

1. If the volume of any single hourly specimen voided during the morning is

greater than the volume of urine voided during the night (10:30 to 7:30 A.M.), the response to the test is negative, that is, such a response indicates the absence of Addison's disease. The authors state that they have not encountered any exceptions to this rule.

2. If the volume of the largest hourly specimen voided during the morning is less than the volume of urine voided during the night, the response to the test is positive, that is, Addison's disease may or may not be present. To establish the diagnosis, Procedure 2 should be instituted.

Procedure 2: (Based on Chemistry of blood and urine)

To carry on with this procedure blood is drawn, preferably under oil (or in a vacutainer tube), while the patient is still fasting, and the plasma analyzed for its content of urea and chloride. The specimen of urine which was voided during the night is also analyzed for urea and chloride. From these four determinations and from the results obtained from Procedure 1 the following equation is solved:

$$A = \frac{\text{urea in urine (mg. \%)}}{\text{urea in plasma (mg. \%)}} \times \frac{\text{Cl}^- \text{ in plasma (mg. \%)}}{\text{Cl}^- \text{ in urine (mg. \%)}} \times \frac{\text{volume of day urine (ml.)}}{\text{volume of night urine (ml.)}}$$

The term "day urine" applied to the largest of the hourly specimens voided during the day; "night urine," to the entire amount which was voided from 10:30 P.M. to 7:30 A.M. It is immaterial how these values are expressed provided that the same method be used throughout the equation. For example, if the concentration of plasma chloride is expressed as mg. of NaCl per 100 ml. the conc. of urinary chloride should be expressed in the same way.

Inferences that may be drawn from Procedure 2: These are as follows:

1. If the value of A in this equation is greater than 25, the patient probably does not have Addison's disease.
2. If the value for this equation is less than 25, the patient probably has Addison's disease provided that renal failure has been excluded.

The authors have encountered low ratios in cases of nephritis, diabetes insipidus and in case of dehydration and fever. With the exception of two cases the question of Addison's disease did not arise. In these two instances, the Cutler-Power-Wilder test excluded Addison's disease. Use of a more complex mathematical expression than the one given has also served to distinguish the response of patients having Addison's disease from those having nephritis.

If the results of Procedure 2 are at all equivocal or if they are not indicative of Addison's disease when there is strong clinical evidence to the contrary, the test devised by Cutler, Power and Wilder may be conducted. This can be instituted immediately. When this is done, none of the patient's time is wasted since the day of the "water test" constitutes the first day of the provocative test. Thus far we have encountered only two instances in which it was necessary to resort to the Cutler-Power-Wilder test and in those cases it also yielded indecisive results.

3. Newman, H.W., and Ashworth, M.J.: A quantitative study on Addison's disease. U.S. Navy Med. Bull. 44, 141 (1949).
4. Ginzburg, L., and Mendenhall, M.: (1954).

Principle:
The sample is reacted in the presence of a reagent which liberates certain volatile organic compounds. The volatile alcohol is then drawn over into and reacts with potassium dichromate as follows:



The reduction of $\text{Cr}_2\text{O}_7^{2-}$ to Cr^{3+} involves a change of color from yellow to green. The degree of color change is compared to a set of known standards.

Apparatus:
See illustration of reaction apparatus page 88a.

Reagents:
1. **Acetic Acid:**
Prepare a 50% (V/V) solution of acetic acid in the usual manner (see stock reagents p. 20). Weigh out 3.333 g. reagent grade potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) dried 24 hours at 110°C . Dissolve the potassium dichromate in about 100 ml. 50% H_2SO_4 in a clean volumetric flask and dilute to the mark with 50% H_2SO_4 . This reagent is stable but keep it protected from direct sunlight.

2. **Sodium Hydroxide:**
Dissolve 1 g. mercuric cyanide, $\text{Hg}(\text{CN})_2$, in 50 ml. distilled water. Add a solution of 15 g. of sodium hydroxide (NaOH) in 50 ml. of distilled water, stirring vigorously. Add slowly with stirring 0.25 g. silver nitrate dissolved in 40 ml. distilled water. The mixture should be clear and may be used immediately. If turbid, allow to stand and decant the clear supernatant for use. This reagent keeps about 2 months.

3. **Light Petroleum (Kerosene):** The silicon detector, Dow Corning A-1-100, may be used.

Procedure:
1. Prepare the reaction apparatus consisting of a train of five tubes as shown in Fig. 81, p. 88a.

ALCOHOL, ETHYL (Blood, Urine, etc.)

References:

1. Bogen, E.: A quantitative study of acute alcoholic intoxication, *Am. J. Med. Sci.* **176**, 153 (1928).
2. Hall, W.W.: Drunkenness. *Navy Medico-legal Aspects*, U.S. Navy Med. Bull. **34**, 149 (1936).
3. Newman, H.W., and Ashenburg, N.J.: A quantitative study on intoxication. *U.S. Navy Med. Bull.* **44**, 744 (1945).
4. Gradwohl, *Legal Medicine*, Mosby Co., (1954).

Principle:

The sample is aerated in the presence of a reagent which fixes certain non-alcoholic reducing agents. The volatilized alcohol is then drawn over into and reacts with potassium dichromate as follows:



The reduction of $Cr^{+6} \longrightarrow Cr^{+3}$ involves a change of color from yellow to green. The degree of color change is compared to a set of known standards.

Apparatus:

See illustration of aeration apparatus page 88a.

Reagents:

1. Anstie's Reagent:

Prepare a 50% (V/V) solution of sulfuric acid in the usual manner (see stock reagents p. 20). Weigh out 3.333 g. reagent grade potassium dichromate ($K_2Cr_2O_7$) dried 24 hours at $110^\circ C$. Dissolve the potassium dichromate in about 900 ml. 50% H_2SO_4 in a liter volumetric flask and dilute to the mark with 50% H_2SO_4 . This reagent is stable but keep it protected from direct sunlight.

2. Scott-Wilson Reagent:

Dissolve 1 g. mercuric cyanide, $Hg(CN)_2$ in 60 ml. distilled water. Add a solution of 18 g. of sodium hydroxide NaOH in 60 ml. of distilled water, stirring vigorously. Add slowly with stirring 0.29 g. silver nitrate dissolved in 40 ml. distilled water. The mixture should be clear and may be used immediately. If turbid, allow to stand and decant the clear supernatant for use. This reagent keeps about 2 months.

3. Liquid Petrolatum (paraffin oil): The silicone defoamer, Dow-Corning Anti-foam A, may be used.

Procedure:

1. Arrange the aeration apparatus consisting of a train of five tubes as shown in Fig. 21, p. 88a.

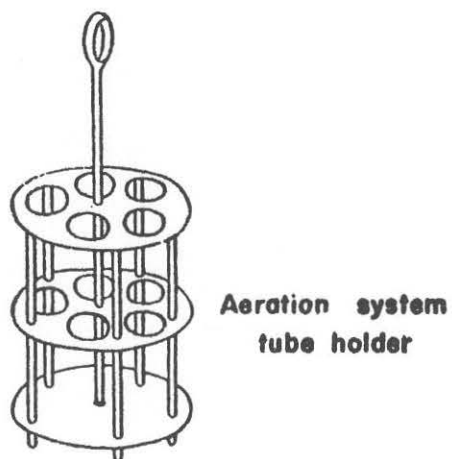
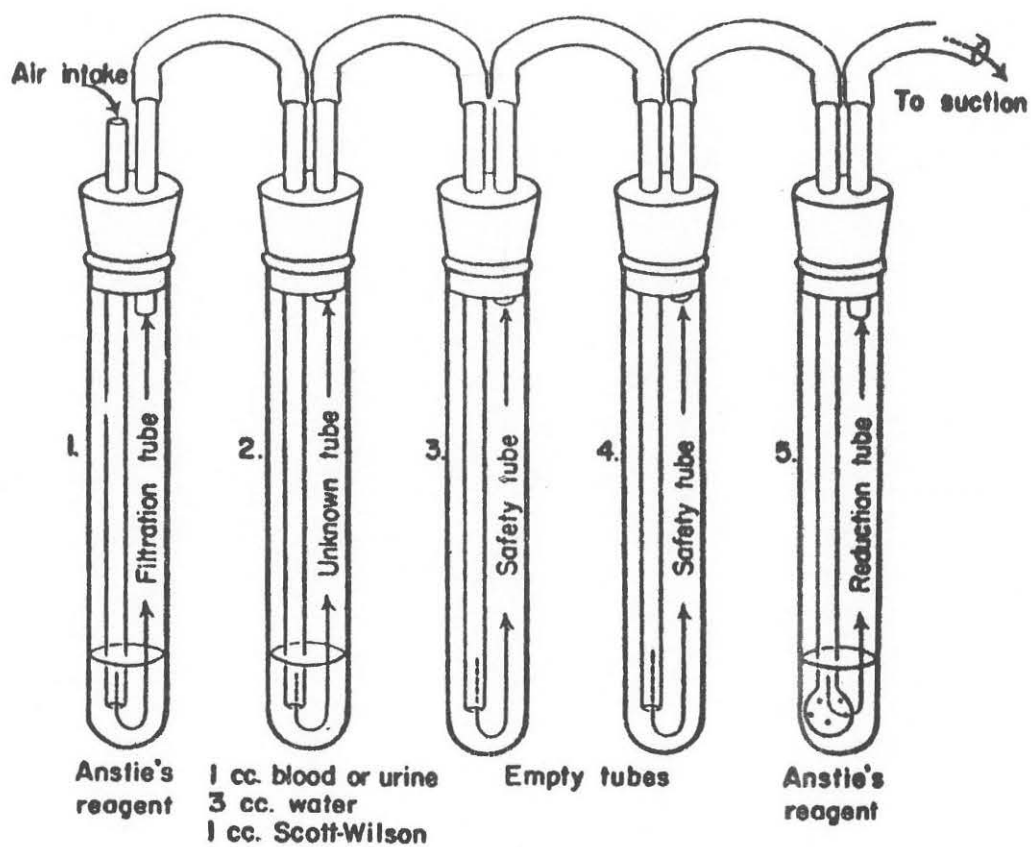


Figure 21. Bogen's blood alcohol apparatus.

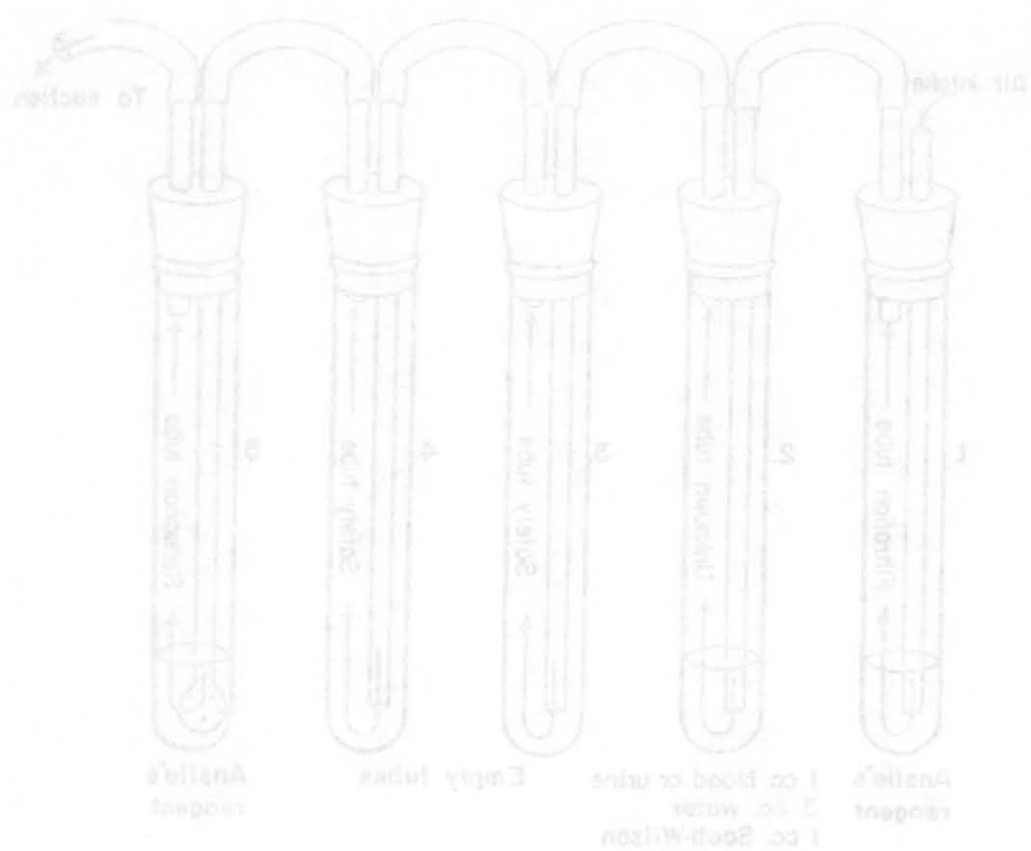


Figure 21. Degen's blood alcohol apparatus.

2. Add to the tubes as follows:

Tube 1 - 5 ml. Anstie's reagent

2 - 1.0 ml. sample (Ostwald pipet)

1.0 ml. Scott-Wilson reagent

4 ml. H_2O

5-10 drops liquid petrolatum or anti-foam

3 - nothing added

4 - nothing added

5 - 5.00 ml. Anstie's reagent (volumetric pipet)

3. Connect all tubes in the train making sure they are appropriately placed.

4. Connect the outlet of the 5th tube to a suction pump for aeration; control the speed of aeration to 2-4 bubbles per second.

5. Immerse the apparatus into a boiling water bath and aerate for 10 minutes keeping the water bath boiling.

6. After aeration period is finished, remove and cool the 5th tube in cold water. Transfer the contents to a clean, dry tube, identical to the standard tubes and dilute up to 5.5 ml. mark with distilled water.

7. Compare the unknown with a known set of standards. Report as mg. alcohol per ml. whole blood.

8. If the alcohol content is 4 mg./ml. or greater repeat the test using 0.5 ml. blood. Multiply the final result by a factor of 2.

Standardization:

Prepare a 1.0% solution of alcohol in water by adding 1.27 ml. absolute alcohol at 15°C. (1.26 ml. at 20°C.) to 50 ml. distilled water in a 100 ml. volumetric flask. Dilute with distilled water to the mark. One ml. standard contains 10 mg. alcohol.

Prepare eleven tubes labeled 0.0 to 5.0 mg. Add to each the desired volume of 1% alcohol solution; e.g., 0.05 ml. to the 0.5 mg. tube - 0.5 ml. to the 5.0 mg. tube. To each tube add 5.00 ml. Anstie's reagent, rinsing the side of the tube during the addition. Add distilled water to each tube to bring the total volume to 5.5 ml. Heat the tubes in a boiling water bath for 10 minutes, remove and cool. Draw and seal the ends of the test tubes in a flame. These standards should be stable for a period of 3 to 4 years.

It is essential for accuracy in this determination that extreme care be taken to use scrupulously clean glassware. Traces of reducing materials such as soap, detergents, proteins, sugars, etc. will interfere in the test by reacting as alcohol does. The Scott-Wilson reagent will unite with most ketones and prevent their interference unless the level is very high.

It is important to avoid the use of alcohol, acetone and ether in the skin preparation for venipuncture in this determination.

It is good practice occasionally to run a blank determination on distilled water to check the reagents.

Other biological fluids may be used instead of blood, such as urine, spinal fluid, and tissue. For spinal fluid analysis see Gettler and Freireich: *J. Biol. Chem.* **92**, 199 (1930). For precautions in the analysis of tissues see Jetter and McLean: *Am. J. Clin. Path.* **13**, 178 (1943) who state that the alcohol level in the body does not appreciably change in the 24-48 hours after death - at least in temperate zones. Actual post-mortem putrefaction may raise the level to as much as 5 mg. per ml.

Interpretation:

Normal persons may show a level in this test as high as 0.5 mg. per 1 ml. of whole blood. The level of brain alcohol lags somewhat behind that of the blood. The individual shows considerable variation in clinical response to a given level of blood alcohol as shown by the following table:

<u>Blood alcohol level of persons suspected of intoxication</u>	<u>% of individuals showing symptoms of intoxication</u>
0.5	10%
1.0	10-18%
1.5	22-47%
2.0	35-84%
2.5	80-90%
3.0	95-100%
4.5	100%

It is an established fact that the intoxicating effect of alcohol like many other drugs is proportional to the amount present in the tissues. In this case the tissue most important is the brain. As the concentration in the blood bears a direct relation to that in the brain, the examination of the blood offers an accurate index of the brain's alcoholic content. The highest blood concentration is usually reached about 1 hour after a given dose. Urine excreted from blood of a given concentration has a concentration equal to or slightly higher than that of the blood. If the concentration of alcohol in blood is 1.00, then urine would be 1.25, and brain 0.90. The breath concentration is also proportional to that of the blood.

It should be remembered that for Navy purposes, drunkenness is defined as "any intoxication sufficient to sensibly impair the rational and full exercise of the mental and physical faculties." Article 112, Manual for Courts Martial.

It may be advisable, in critical medico-legal examinations, to run simultaneous Bogen's blood alcohol determinations on the patient and on the analyst, so as to eliminate the possibility of contamination.

ALCOHOL - METHYL Serum

Reference:

Osburn, E. E.: U. S. Naval Medical Bulletin 46, 1170 (1946)

Principle:

A protein-free filtrate of blood or other protein-free material is treated with an oxidizing agent (at room temperature to differentiate from other substances such as ethyl alcohol) to convert methyl alcohol to formaldehyde. Chromotropic acid (1, 8 dihydroxynaphthalene - 3, 6 disulfonic acid) reacts with formaldehyde in the presence of sulfuric acid and heat to give a purple color.

If chromotropic acid is not available a Leach test may be performed using milk - see below. This is a somewhat less sensitive test.

Reagents:

1. 20% (w/v) Trichloroacetic acid. Dissolve 20 g. $\text{CCl}_3 \cdot \text{COOH}$ up to 100 ml. total volume. Mix well. Store in refrigerator.
2. Acid potassium permanganate. To 3 g. KMnO_4 , add 25 ml. phosphoric acid (H_3PO_4 - 85% anal. reagent), dilute up to 100 ml. Shake until the potassium permanganate is dissolved.
3. Sodium bisulfite (NaHSO_3) solid, powdered anal. reagent.
4. Chromotropic acid (1, 8³- dihydroxynaphthalene - 3, 6 disulfonic acid) solid, powdered. Eastman Kodak # P230 (sodium salt).

Procedure:

1. Using a serological pipet, place 2 ml. of oxalated blood in a 25 x 200 mm. test tube.
2. Add slowly with shaking 2 ml. of 20 per cent trichloroacetic acid. Stopper and shake well for 1 minute.
3. Transfer to a centrifuge tube and centrifuge at 2,000 r.p.m. for 5 minutes.
4. Pipet 1 ml. of the clear supernatant fluid into a test tube. (If the supernatant fluid is turbid, filter it through a small retentive filter paper.)
5. Add 0.2 ml. of the potassium permanganate reagent. Mix and let stand for 1 minute.
6. Add about 10 mg. of powdered sodium bisulfite to decolorize completely the solution of permanganate.
7. To the water-clear solution, add a small amount of chromotropic acid powder and shake. (Use approximately the amount of chromotropic acid that will adhere to the end of a safety match.)
8. Add 1.5 ml. of concentrated sulfuric acid, allowing the acid to flow down the side of the tilted tube to form a separate layer in the bottom of the tube.
9. Examine the junction of the two layers against a white background. If methyl alcohol is present, a purple ring will usually be noted.
10. Cautiously mix the contents of the tube by gently twirling, and allow the tube

to stand until the mixture has reached room temperature. If methyl alcohol was present in the original fluid, a diffuse violet color will be noted.

11. It is advisable to perform a positive test by adding a drop of methyl alcohol to 2 cc. of normal blood and complete the procedure. Furthermore, a negative control of the unknown should be run by omitting the chromotropic acid in step 7 and continuing the procedure. Suspected beverages may be tested for methyl alcohol by the same procedure as was used for blood or body fluids.

12. In order to be sure that pre-formed formaldehyde (or a precursor such as methenamine, a drug which liberates formaldehyde) is not present, a test should also be run omitting the oxidation (Steps 5 and 6).

13. If chromotropic acid is not available, the Leach test may be run as follows:

To 10 ml. of milk (fresh, skim, or reconstituted), add 1 ml. of the filtrate obtained in Step 6 above. Add 10 ml. of concentrated (37%) HCl containing 0.02% FeCl_3 . Heat to boiling. A violet color indicates the presence of formaldehyde. The positive and negative controls indicated in Steps 11 and 12 should also be run with this test.

Calculation:

This is a qualitative test. A rough semi-quantitation may be made by adding various known amounts of methyl alcohol to blood and comparing the unknown with these.

Interpretation:

The actual level of methyl alcohol has little relationship to clinical toxicity. Methyl alcohol is metabolized in the body to formic acid ($\text{H} \cdot \text{COOH}$) which is the actual toxic agent. One of the very serious effects of formic acid toxicity is its effect on the optic nerve resulting in blindness which is more or less complete and may be permanent.

AMINO-ACIDS In Plasma and Urine

References:

- Shroeder, W.A., Kay, L.M., and Mills, R.D.: *Anal. Chem.* **22**, 760 (1950).
Albanese, A.A., and Irby, V.: *J. Lab. Clin. Med.* **30**, 718 (1945).
Albanese, A.A., and Irby, V.: *J. Biol. Chem.* **153**, 583 (1944).
Fister, H.J., *Manual of Standardized Procedures*, Standard Scientific Supply Co. (1950).

Principle:

Amino-acids in neutral solution react with alkaline copper phosphate to form soluble cupric salts usually of the form A_2Cu . After the excess copper phosphate is removed the soluble copper in the filtrate is determined by iodimetric titration. Ammonia does not contribute to the copper value nor does creatine, creatinine, uric acid or urea. However, dipeptides, tripeptides and in general the "end-group" amino acids with a free carboxyl adjacent to a peptide-linked nitrogen or with a free amino group adjacent to a peptide-linked carboxyl group. It has been shown to include urine citrate also.

Apparatus:

Micro-burets

- 10 ml. (or less) for urine
- 4 ml. (or less) for plasma

Reagents:

1. Cupric chloride - 28 g. of reagent grade $CuCl_2 \cdot 2H_2O$ per liter of water solution.
2. Sodium phosphate - 68.5 g. of reagent grade $Na_3PO_4 \cdot 12H_2O$ per liter of water solution.
3. Sodium tetra-borate buffer - pH 9.1 to 9.2. 40.3 g. of reagent grade anhydrous $Na_2B_4O_7$ (or 76.4 $Na_2B_4O_7 \cdot 10H_2O$) are dissolved in 4 liters of water and filtered.
4. Washed copper phosphate - To 40 ml. of sodium phosphate solution is added 20 ml. of cupric chloride solution with swirling. The suspension is now centrifuged for 5 minutes. The precipitated copper phosphate is washed twice by resuspension in 60 ml. of sodium tetra-borate solution followed by recentrifuging. The washed precipitate is suspended in 100 ml. sodium tetraborate solution and 6 g. of reagent grade sodium chloride (NaCl) is dissolved in the suspension. The suspension is stable in glass-stoppered flasks up to 4 to 10 days.
5. Thymolphthalein indicator - 0.25 g. of thymolphthalein indicator is dissolved in 75 ml. absolute methyl alcohol (or 50 ml. 95% ethyl alcohol) and diluted to 100 ml. with water.
6. Sodium thiosulfate 0.1N - Dissolve 25 g. $Na_2S_2O_3 \cdot 5H_2O$ in 200 ml. distilled, boiled, and cooled water. Add and dissolve 11.4 g. borax $Na_2B_4O_7 \cdot 10H_2O$ (or 6.0 g. anhydrous $Na_2B_4O_7$) and 15 ml. iso-amyl-alcohol (the latter requires considerable water to dissolve). Dilute to one liter and mix well.

7. Potassium iodate standard - Dissolve 0.3567 g. dry KIO_3 up to 1 liter with water. Use to standardize the 0.01N and 0.001N sodium thiosulfate solutions (using 10.00 ml. and 1.00 ml. respectively).

8. Starch Indicator - Dissolve 1 g. Lintner soluble indicator starch in 100 ml. of saturated NaCl by heating on the steam bath, cool overnight, and decant the supernatant solution.

9. Potassium iodide - Prepare just before use. 10 g. analytical reagent KI dissolved up to 10 ml. with water.

10. Glacial acetic acid - analytical reagent.

11. NaOH, 1N. 40 g. diluted up to one liter with water.

12. Trichloroacetic acid 10% w/v

Procedure:

Urine

Preservation - 24-hour specimens are collected in brown bottles containing 50 ml. HCl (dilute 15 ml. anal. reagent conc. HCl up to 100 ml. with water) and 1 ml. of 10% alcoholic thymol solution. The total volume is made up to 2,000 ml. and mixed well before removal of samples. Amino acids in urine are stable up to one week at room temperature.

Method - To 15 ml. of urine sample in a 50 ml. volumetric flask are added 4 drops of thymolphthalein indicator and 1N NaOH to a faint green or blue color. Then 30 ml. of copper phosphate suspension are added from a graduated cylinder and the volume is brought to the mark with distilled water. Mix thoroughly by repeated, complete inversion and allow to stand for five minutes. Filter through No. 5 Whatman (similar) paper filters into 125 ml. Erlenmeyer flasks.

The copper content of 10.00 ml. aliquots of the filtrate is determined as follows: Each aliquot is acidified by 0.5 ml. glacial acetic acid. To this acidified solution is added 1 ml. KI solution. The solutions are then titrated with standardized 0.01 N sodium thiosulfate from a 10 ml. micro-burette. Six drops of starch indicator are added when the solution is a very light yellow and the mixture is titrated to a colorless end-point.

Calculation:

Since two moles. of amino nitrogen combine with one copper atom, each ml. of 0.01N thiosulfate is equivalent to 0.28 mg. amino nitrogen. Assuming exactly 0.01N thiosulfate was used to titrate 10.00 ml. of filtrate then -

$$\text{ml. thiosulfate} \times \frac{0.28}{3} = \text{mg. amino N/ml. of sample}$$

Example: A 24-hour collection of urine was brought to a total volume of 2000 ml. and treated as above. Two 10.00 ml. aliquots were titrated and a standardization was carried out and the following data collected.

9.79 ml. of approximately 0.01 N sodium thiosulfate was required to titrate 10.00 ml. of 0.01 N standard potassium iodate.

$$\underline{N \text{ of thiosulfate}} = \frac{10.00}{9.79} \times 0.01 = 0.01021 \underline{N}$$

$$f = 1.021$$

Two 10.00 ml. filtrate aliquots (each representing 3.00 ml. of original sample) required 2.10 and 2.12 ml. for titration:

$$\begin{aligned} \text{amino N/ml. original sample} &= 2.11 \times 1.021 \times \frac{0.28}{3} \\ &= 0.2015 \end{aligned}$$

$$\text{amino N/24 hours} = 0.2015 \times 2000 = 403.0 \text{ mg.}$$

Plasma or Serum

Method - In a 15 ml. centrifuge tube place 3.00 ml. 10% trichloroacetic acid. Add dropwise with mixing 1.00 ml. of plasma or serum. Mix thoroughly and allow to stand for 10 minutes, then centrifuge for 5 minutes. The supernatant solutions are decanted through Whatman #5 filter paper into test tubes and 2.00 ml. aliquots removed to graduated (at 10 ml.) 15 ml. conical centrifuge tubes.

To each tube is added in succession one drop of thymolphthalein indicator, NaOH, 1 N, to the appearance of a blue color, 5 ml. of cupric phosphate suspension and distilled water to the 10 ml. graduation.

After being mixed by vigorous shaking, the reaction mixtures are allowed to stand for 5 minutes and are then centrifuged for 5 minutes. 5.00 ml. aliquots of the supernatant are pipetted into 125 ml. Erlenmeyer flasks. They are acidified with 0.5 ml. glacial acetic acid. Then 1.0 ml. of KI solution and 3 drops of starch indicator solution are added and the sample titrated with standardized 0.001 N sodium thiosulfate from a very fine tipped 5 ml. microburette to the disappearance of the blue color.

Calculations:

Each ml. of 0.001 N thiosulfate is equivalent to 0.028 mg. amino N. Therefore since the final sample represents 0.25 ml. serum:

$$\text{mg. amino N per 100 ml. serum} = \text{ml. thiosulfate} \times \text{factor} \times 0.028 \times \frac{100}{0.25}$$

Example: In the standardization of the 0.001 N sodium thiosulfate 9.42 ml. were required to titrate 1.00 ml. of 0.01 N KIO₃. (The factor therefore was $10 \div 9.42 = 1.062$.)

5 ml. of final filtrate obtained as directed above required 0.65 ml. of this sodium thiosulfate for titration, therefore: $\text{mg\% amino N} = 0.65 \times 1.062 \times 0.028 \times \frac{100}{0.25} = 7.74$

Standardization

0.01 N and 0.001 N $\text{Na}_2\text{S}_2\text{O}_3$ are prepared from 0.1 N (see stock reagents) by dilution with distilled water using volumetric pipets and flasks.

0.01 N $\text{Na}_2\text{S}_2\text{O}_3$

To 10 ml. of 0.01 N KIO_3 , add about 4 ml. of water, 0.5 ml. glacial acetic acid, 1 ml. KI solution, and titrate with 0.01 N $\text{Na}_2\text{S}_2\text{O}_3$ to a very faint yellow. Add six drops of starch indicator and continue the titration to a complete disappearance of the blue color. Calculate a factor. See p. 30.

0.001 N $\text{Na}_2\text{S}_2\text{O}_3$

To 1.00 ml. of 0.01 N KIO_3 add about 4 ml. of water, 0.5 ml. glacial acetic acid, 1 ml. KI solution, and 3 drops of starch indicator. Titrate with 0.001 N $\text{Na}_2\text{S}_2\text{O}_3$ to the complete disappearance of the blue color. Calculate a factor. See p. 30.

Notes:

The analysis on serum or plasma must be carried out promptly. Hemolysis results in markedly elevated values. Fresh urine specimens must be used or they may be preserved as outlined above.

Interpretation:

Urine amino nitrogen values by this method range from 200 to 700 mg. per 24 hour period which is 3-4 per cent of the total nitrogen content of the urine.

Increases in urine amino acids are seen in (1) acute severe hepatic injury; (cystinuria (a congenital renal tubular defect involving lysine and arginine); (3) Fanconi syndrome in which renal glycosuria and renal amino aciduria are both present; (4) Wilson's disease which is hepatolenticular degeneration associated with a defect in copper metabolism.

It is important to determine the amino N of urine in terms of per cent of total nitrogen. Normally between 3-4 per cent by this method - it may rise to 15 per cent in a Fanconi syndrome.

Plasma levels are a little lower than serum levels, apparently because of reactive peptides released from fibrinogen during coagulation, and range between 4-8 mg. %.

In severe liver damage, values as high as 200 mg. % have been reported. A decrease is seen after the injection of insulin.

For specific identification of amino acids, paper chromatography is used. See Dent, C. E. and Shilling, J. A.: *Biochemistry Journal*, **44**, 318 (1948).

AMYLASE

Serum, Plasma, Etc.

References:

- (1) Somogyi, M.: The Estimation of Diastase. J. Biol. Chem., 125, 399 (1938).
- (2) Somogyi, M.: A New Sugar Reagent. J. Biol. Chem., 160, 66 (1945).
- (3) Nelson, N.: J. Biol. Chem., 153, 375 (1944).
- (4) Smith, B.W. and Roe, J.H.: J. Biol. Chem., 179, 53 (1949).
- (5) Peralta, O. and Reinhold, J.G.: Clinical Chem. 1, 157 (1955).

Principle:

The amylase is allowed to act on the substrate (cornstarch) for a period of time after which the reaction is stopped. The extent of hydrolysis is then measured by determining the increase in reducing substances (sugars). The reducing substances may be determined by titrimetric (Ref. 1) methods or colorimetric procedures (Ref. 2 and 3). Alternatively, the decrease in substrate (starch) concentration may be measured by the decrease in iodine-starch complex color (Ref. 4) or in turbidity of the starch solution (Ref. 5). Only the colorimetric method will be described here.

The maltose (reducing sugar), liberated by the action of amylase on the starch substrate, is measured by the blood glucose method (p. 181). Because maltose is less active as a reducing agent, the period of heating with the alkaline copper reagent is increased to twenty minutes instead of ten. Because of the presence of glucose and other reducing substances in the serum-starch mixture before incubation, a zero-time blank is necessary. After reduction, color development, measurement and calculations are carried out as for glucose in blood (p. 181).

Apparatus:

Colorimeter or photometer (500-550 mμ). Folin-Wu (or similar) sugar tubes 25 ml.

Reagents:

1. Starch paste. Grind thoroughly in a mortar 3 grams of washed starch with 10 ml. of water and pour into 180 ml. of boiling water. Rinse the mortar with 10 ml. more of water. Boil one minute with agitation. Then cover the mouth of the flask with a beaker and set in a boiling water bath for 15 to 30 minutes. Then cool. Store under refrigeration.

2. Acid NaCl. 10 grams NaCl is added to 3 ml. 0.1 N HCl, dissolved in 500 ml. water and diluted to one liter.

3. Protein precipitants

a. 5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$

b. 6% sodium tungstate

4. Alkaline copper reagent

See under blood glucose (p. 181).

5. Arseno-molybdate (Nelson color reagent)

See under blood glucose (p. 181).

Procedure:

1. For zero-time control

a. To a 16 x 120 mm. pyrex test tube add exactly 5.0 ml. starch paste, 2.0 ml. acid NaCl and 1.0 ml. 5% copper sulfate.

b. Add 1.00 ml. serum and mix.

c. Add 1.0 ml. 6% sodium tungstate solution, mix well, centrifuge and/or filter through a quantitative filter paper.

2. For enzyme activity test

a. To a 16 x 120 mm. pyrex test tube add exactly 5.0 ml. starch paste, 2.0 ml. acid-NaCl and warm to 40°C., then add 1.00 ml. serum, mix.

b. Incubate exactly 30 minutes at 40°C.

c. Immediately add 1.0 ml. 5% CuSO_4 and 1.0 ml. 6% sodium tungstate, mixing after each addition. Continue as for control tube in preparing a filtrate.

Colorimetric Procedures:

Introduce into:

1. Sugar tube C - 1.0 ml. control filtrate
2. Sugar tube E - 1.0 ml. enzyme filtrate
3. Sugar tube B - 1.0 ml. distilled water (reagent blank)
4. Sugar tube S - 1.0 ml. 20 mg. % glucose standard (nominal 200 mg. % standard)

To each of the four tubes add 1.0 ml. alkaline copper reagent and mix well. Heat in a boiling water bath 20 minutes. Remove and cool in water bath. Add 1.0 ml. color reagent, mix well until all cuprous oxide is dissolved. Dilute to 25 ml. mark. Mix well by inversion. Compare photometrically (515 mμ) as in the blood glucose method (p. 182) setting the reagent blank (B) at 100% T (D = 0).

Calculation:

Amylase activity is expressed as mg. glucose equivalents liberated in 1/2 hour per 100 ml. serum. Since the control tube also shows some reduction:

$E - C$ = amylase activity expressed as glucose equivalents (Somogyi units)

The term glucose equivalents is used to denote the fact that whereas maltose is the actual product of amylolytic activity, glucose is the standard against which it is determined.

Example: Tube C - gave a reading of 63.0% transmittance
Tube E - gave a reading of 31.4% transmittance
Tube B - was set at 100% transmittance
Tube S - gave a reading of 25.0% transmittance

corresponding to the following optical densities

C - 0.201
E - 0.503
B - 0.000
S - 0.602

Using the usual photometric formula

$$Cu = (Du/D_s) C_s \times \frac{100}{0.1} \quad \text{see Glucose-blood (p. 182).}$$

$$C_C = 66.6$$

$$C_E = 167$$

$$\text{Then } E - C = 100 \text{ mg. \%} = 100 \text{ Somogyi units}$$

Notes:

On fluids with high activity, it is more accurate to make a dilution with 0.85% NaCl before incubation rather than after.

Interpretation:

Normal range - 40-120 Somogyi units is the usual range although apparently some normals range up to 200 units.

Significance -

Elevations - are seen in acute pancreatic disease - less marked elevations are seen in chronic disease of pancreas.

Decreases - are seen in liver disease.

Acute pancreatitis - elevations may exceed 1000 units and may fall rapidly to normal values in 3-4 days.

Chronic pancreatitis - increases over base line values of 100-200 units.

Other abdominal diseases such as:

- (1) Intestinal obstruction
- (2) Acute peritonitis
- (3) Perforated peptic ulcer

In these, smaller increases not more than 500 units are seen developing 5-10 days after the onset of the symptoms and appear to be due to increased pressure on the pancreatic duct.

Increases are also seen in mumps and obstructive disease of the salivary glands.

AMYLASE

Serum

Reference:

- Gomori, G., *Am. J. Clin. Path.* **27**, 714 (1957)
Huggins, C., and Russell, P.S., *Ann. Surg.*, **128**, 668 (1948)
Smith, B.W., and Roe, J.H., *J. Biol. Chem.*, **227**, 357 (1957)

Principle:

A starch substrate is acted on by the amylase in serum. The decrease in the blue starch-iodine color is measured photometrically.

Reagents:

1. Starch substrate. Dissolve 8 g. of Lintner's soluble starch (Merck) and 1 g. benzoic acid in 800 ml. of boiling distilled water. Cool. Dilute up to 1000 ml. with distilled water. Mix well. This solution may be kept at room temperature and should be stable at least six months.

2. 5% sulfuric acid. Dilute about 27 ml. of concentrated sulfuric acid up to one liter with distilled water. Cool.

3. Iodine solution. Dissolve 1 g. of I_2 and 2 g. of KI in 50 ml. of distilled water. Dilute up to 300 ml. with distilled water.

4. Phosphate buffer 0.1 M pH 7.4
 $NaH_2PO_4 \cdot H_2O$ 2.30 g.) Dissolve, dilute to one liter and mix.
 Na_2HPO_4 anhyd. 11.83 g.) Check pH with glass electrode and standard buffer.

Procedure:

Into 3 large tubes calibrated at 50 ml., (T-test; C-control; and B-blank) place solutions and proceed as indicated.

<u>Solutions</u>	<u>Tubes</u>	<u>B</u>	<u>C</u>	<u>T</u>
Starch substrate	---	0.5	0.5	
Buffer	---	0.5	0.5	
Serum	---	---	0.1	
<hr/>				
Mix and incubate at 37°C. for 30 minutes.				
<hr/>				
Sulfuric acid 5%	2	2	2	
Iodine solution	1	1	1	
<hr/>				
Mix well, dilute to 50 ml. with distilled water.				

Compare photometrically at 640 mu using the blank to set at 100% T.

Standardization:

1. Prepare a dilution of the starch substrate by diluting 10 ml. to 100 ml. with distilled water.

2. Into 50 ml. volumetric flasks pipet diluted starch as follows:

Flask #	ml. diluted starch	% of control	mg. starch
1	0.0	0	0
2	0.5	10	0.4
3	1.0	20	0.8
4	2.0	40	1.6
5	3.0	60	2.4
6	4.0	80	3.2
7	5.0	100	4.0

3. Add 2 ml. of sulfuric acid 5% and 1 ml. of iodine solution. Mix. Dilute to the mark and mix well.

4. Compare photometrically at 640 mu using the blank to set at 100% T.

5. Plot the O.D. vs. mg. starch and determine the optical density due to 1 mg. of starch.

Calculations:

$$\frac{D_{\text{control}} - D_{\text{test}}}{D_{1 \text{ mg.}}} \times 100 = \text{amylase units.}$$

Note:

If the starch is completely decolorized or if a value of more than 400 units is found, the test should be repeated with a dilution of serum. Although the control contains 4 mg of starch, this amount may be tripled and subsequently diluted to the factor.

ASCORBIC ACID

Blood, Urine and Tissue

Reference:

- (1) Roe, J. H. and Kuether, C. A., J. Biol. Chem., 147, 399 (1943)
- (2) Shaffert, R. R., and Kingsley, G. R., J. Biol. Chem., 212, 59 (1955)

Principle:

Ascorbic acid in blood or urine filtrate is oxidized by means of activated charcoal (Norit) to dehydroascorbic acid (reversible oxidized ascorbic acid) which is then reacted with 2, 4-dinitrophenylhydrazine to form a hydrazone. (Thiourea is added to prevent interference by oxidizing substances such as Fe^{+++} or H_2O_2 which produce a color with 2, 4-dinitrophenylhydrazine.) The hydrazone derivative is now treated with strong sulfuric acid to produce a reddish color which is measured photometrically at a wavelength of 540 millimicrons.

This procedure measures total ascorbic acid (reduced ascorbic acid plus dehydroascorbic acid) which may be partitioned by running two parallel determinations and omitting the activated charcoal treatment in one of them. Only dehydroascorbic acid is measured when this step is omitted.

Reagents:

Stock ascorbic acid standard - Accurately weigh exactly 100 mg. of L-ascorbic acid and place in a 100 ml. volumetric flask. Dilute to volume with 4 per cent trichloroacetic acid solution.

Working ascorbic acid standard - Dilute 2 ml. of stock standard to 100 ml. with 4 per cent trichloroacetic acid. 1 ml. = 20 micrograms of L-ascorbic acid.

Trichloroacetic acid - 4 per cent solution and 6 per cent solution. (W/V).

2, 4-Dinitrophenylhydrazine - 2 g. of 2, 4-dinitrophenylhydrazine (Eastman) are dissolved in 100 ml. of 9 N Sulfuric acid. Let stand overnight and filter through Whatman No. 42 filter paper.

9 N sulfuric acid - Prepare by adding one part of concentrated H_2SO_4 to 3 parts of water (V/V).

85 per cent sulfuric acid - To 100 ml. of water add 900 ml. of concentrated sulfuric acid.

Thiourea - Dissolve 10 g. of thiourea (Eastman) in 55 ml. of 95% ethyl alcohol and

dilute to 100 ml. with distilled water. This reagent should keep 2 months.

Norit (activated charcoal) - Use U.S.P. grade. If the blanks read too high, add 1 liter of 10 per cent hydrochloric acid (1 part concentrated HCl to 9 parts water) to 200 g. of Norit in a large flask, bring to a boil, and filter with suction. Stir the cake with 1 liter of water and again filter. Dry the cake in an oven overnight at 100-120°C. Repeat if necessary.

Oxalic acid - 0.5 per cent (W/V) solution.

Procedure:

Standardization: Shake vigorously 25 ml. of working standard L-ascorbic acid with 1/2 teaspoon (0.7 g.) of Norit for 1 minute. Filter through Whatman No. 42 filter paper. Add 0.0, 0.25, 0.50, 1.0, 1.5, 2.0, 2.5, and 3.0 ml. of filtrate to photometric cuvetts and dilute each to 4 ml. with 4 per cent trichloroacetic acid which has also been shaken with Norit and filtered. Add 1 drop of thiourea solution and 1 ml. of 2, 4-dinitrophenylhydrazine solution. Continue as under Development of Color, Step 2.

Preparation of Filtrate:

Whole blood and serum:

- (1) To 15 ml. of 6 per cent trichloroacetic acid in a 50 ml. Erlenmeyer flask, add dropwise 5.00 ml. of sample mixing continuously.
- (2) Let stand for 5 minutes and centrifuge for 10 minutes at 2500 rpm.
- (3) Pour off the supernatant into a clean, dry test tube, add 1/2 teaspoon of Norit, and shake vigorously for 1 minute. Filter through Whatman No. 42 filter paper.

Urine:

- (1) Add 1 ml. of urine to 19 ml. of 4 per cent trichloroacetic acid. Add 1/2 teaspoon of Norit and shake vigorously for 1 minute.
- (2) Filter through Whatman No. 42 filter paper.

Development of Color:

1. To 4 ml. of filtrate in a photometric cuvet, add 1 drop of thiourea solution and 1 ml. of 2, 4-dinitrophenylhydrazine solution.

2. Place the tube in a boiling water bath exactly 5 minutes for blood or serum and 10 minutes for urine and standards. At the end of this time place the tube in crushed ice for 5 minutes.

3. Add slowly (drop by drop) 5 ml. of 85 per cent sulfuric acid, and mix by twirling. Let stand for 10 minutes at room temperature. Read in the photometer (540 mμ) against its own blank, prepared in exactly the same manner, except for the omission of the 2,4-dinitrophenylhydrazine until after the addition of the 85 per cent sulfuric acid.

Calculation

Blood or serum:

$$(D_u/D_s) \times C_s \times (100/1) = \text{ascorbic acid in mg./100 ml.}$$

Using the 1.5 ml. standard (containing 30 ug. ascorbic acid):

$$(D_u/D_s) \times 0.03 \times 100 = \text{mg. \% ascorbic acid.}$$

Urine: (Using the 1.5 ml. standard)

$$(D_u/D_s) \times 0.03 \times (1000/0.2) = \text{mg. ascorbic acid per liter of urine.}$$

Example: A sample of whole blood treated as indicated and read against its own blank showed a transmittance of 72 per cent. The 1.5 ml. standard (0.03 mg.) read against its own blank, gave a transmittance of 32 per cent.

	T (%)	O. D.
Unknown	72	.143
Standard	32	.495

$$(0.143/0.495) \times 0.03 \times 100 = 0.87 \text{ mg. ascorbic acid per 100 ml. whole blood.}$$

Notes

Reductones (degradation products of sugars) and diketogulonic acid (a product of intra-molecular rearrangement of dehydroascorbic acid) as well as certain other aldehydes and ketones, also give hydrazones with 2,4-dinitrophenylhydrazine. The interference is slight for all these except for diketo-gulonic acid because in the strong acid these products absorb only very slightly at the chosen wavelength (540 millimicrons). Diketo-gulonic acid will be read as if it were dehydroascorbic acid. Its level in normal blood and urine samples is close to zero. In tissues it may be a significant fraction of the total ascorbic acid content. For a complete fractionation of these compounds see Roe, J.H., et al., J. Biol. Chem., 174, 201 (1948).

Interpretation

The normal range for whole blood total ascorbic acid is 1.2 to 2.3 mg. per 100 ml. In serum the normal range is 0.5 to 1.4 mg. per 100 ml. Reduced ascorbic acid present

originally (omitting the Norit treatment) is somewhat lower.

In acute or recent ascorbic acid deprivation (2-3 weeks) the plasma or serum level may be close to zero but the whole blood levels will still be in the normal range. The white blood cells are the last to lose their ascorbic acid in deprivation studies.

ASCORBIC ACID SATURATION TEST

Principle:

It is assumed that the tissues of a person with a previous history of insufficient ascorbic acid intake will take up quite large amounts when a test dose is given so that relatively little will be excreted in the urine.

Various dosages, both oral and intravenous have been used, and various periods of urine collection have been chosen. It is recommended that the test be run in parallel with a person known to have a history of satisfactory ascorbic acid intake.

Procedure:

10 A.M. Give orally 10 mg. ascorbic acid per kilogram body weight.

2 P.M. Empty the bladder completely and discard the urine.

4 P.M. Empty the bladder completely and determine the total ascorbic acid excreted in the two-hour period.

Repeat the test daily until normal results are obtained. Ordinary meals may be consumed.

Interpretation:

In normal persons an excretion of about 50 mg. (during the two-hour period) will be obtained on the first or second day. When a vitamin C deficiency is present, a longer time, six to ten days, or up to several weeks may be required.

The test has the obvious advantage of serving both as therapy and as a test. Progress in therapy is indicated by the increased amount of ascorbic excreted as time goes on.

BILIRUBIN

In Serum

References:

1. Malloy, H. T., and Evelyn, K. A., J. Biol. Chem. **119**, 481-90 (1937).
2. Ducci, H., and Watson, C. J., J. Lab. Clin. Med. **30**, 293 (1945).
3. Zieve, Hill, Hansom, Falcone, and Watson, J. Lab. Clin. Med. **38**, 446 (1951).
4. Altman, V., Nelson, M., and Pernova, D., Am. J. Clin. Path. **26**, 956 (1956).

Principle:

Bilirubin, like aromatic amines, combines with diazonium salts to form azo dyes. In this case, sulfanilic acid is diazotized with nitrous acid ($\text{HCl} + \text{NaNO}_2$) and is then coupled to bilirubin. The distinction between direct one-minute (1'B) and total (TB) bilirubin depends upon the fact that addition of alcohol allows all the bilirubin to react with the diazo reagent and is used to make the reaction quantitative for total bilirubin content of the serum. Apparently there are two types of bilirubin: direct-reacting, polar, water soluble; and indirect-reacting, non-polar, alcohol soluble.

Recently, Schmid has shown that the polar, water soluble, direct-reacting bilirubin is bilirubin glucuronate which is formed by a conjugation process in the liver from free bilirubin which is the non-polar indirect-reacting type of bilirubin.

Reagents:

1. Methyl alcohol-absolute (or redistilled).
2. Sulfanilic acid solution. Dissolve 1 g. of sulfanilic acid in 1 liter of dilute HCl (15 ml. concentrated HCl diluted to 1 liter).
3. Sodium nitrite solution: Dissolve 0.5 g. NaNO_2 (ACS) in a little water, dilute to 100 ml. Prepare on day of use (or prepare from stock refrigerated 50% on day of use).
4. Diazo reagent. Add 0.3 ml. of the nitrite solution to 10 ml. of the sulfanilic acid solution. Mix. Prepare mixture within 5 minutes of time of use.
5. Dilute HCl for blanks. Dilute 15 ml. of concentrated HCl to 1 liter with water.
6. Bilirubin stock standard. Transfer exactly 20 mgm. of pure bilirubin to a 100 ml. glass stoppered volumetric flask. Dissolve. Bring to mark with chloroform and mix. This solution is stable in the dark and cold, with well fitting stopper.
7. Bilirubin working standard. Dilute 10 ml. of the above standard to 100 ml. with ethyl alcohol 95%. This results in a concentration of 2 mg./100 ml.

Procedure:

Collection of specimen:

1. Post-absorptive to avoid lipemia.
2. Plain tube (serum).
3. Run test within 2 hours after clotting. After long standing only the indirect reaction occurs.
4. Use filter or wavelength indicated in calibration chart (530-560).

Analysis of Serum: (Note the necessity for step timing for direct bilirubin!)

1. Pipette exactly 2.0 ml. of serum into a clean dry test tube, add exactly 8.00 ml. of distilled H₂O, stopper and mix thoroughly.
2. Pipette into each of two test-tube cuvetts, a 4 ml. portion of this 1:5 dilution of the serum.
3. Add to one tube 1 ml. of diazo blank solution, and to the other 1 ml. of the freshly prepared diazo reagent, mixing well.
4. After exactly one minute set the blank at 100.0 and read the unknown.
5. Add 5.0 ml. of methyl alcohol absolute. Mix well, allow to stand 30 minutes for full color to develop. Read again as in step 4.

Calibration curve:

Prepare a calibration curve using 0.0, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0 ml. of dilute working standard and ethyl alcohol to make 9.0 ml. Add 1.0 ml. diazo reagent, mix well. Read after 30 minutes. These standards correspond to up to 10 mg. per 100 ml. for the direct and up to 20 mg. per 100 ml. for the total bilirubin.

Calculation:

1. Direct (1'B) bilirubin

$$C_u \text{ (mg. \%)} = (D_u/D_s) \times C_s \times (100/1) \times (V_u/V_s) \text{ see page 57}$$

If $(C_s/D_s) \times 100 = K$ and since $V_u = 5 \text{ ml.}$ and $V_s = 10 \text{ ml.}$

$$\text{then } C_u = D_u \times K \times V_u/V_s ;$$

$$C_u = D_u \times (K/2)$$

2. Total (TB) bilirubin ($V_u = 10$ and $V_s = 10$)

$$C_u = D_u \times K$$

3. Micro-method (total bilirubin) see p. 109

$$C_u = (D_u/D_s) \times C_s \times (100/0.05) \times (2.5/10)$$

$$C_u = D_u \times 5K$$

Interpretation:

Watson places the upper limit of normal 1'B at 0.25 mg. % and the upper limit of TB at 1.50 mg. %. An increase mainly in the indirect reacting fraction indicates hemolytic jaundice, while an increase in the direct reacting indicates an hepatic or post-hepatic cause of the jaundice.

The direct and indirect reacting bilirubin are not completely separated by this procedure. Only about 65% of the direct-reacting bilirubin is measured at one minute; practically all will be measured at 15 minutes. At this time however, some of the indirect-reacting bilirubin has started to react. Zieve et al, with Watson, found that the 1'B gave the best clinical differentiation between the obstructive and the hemolytic types of jaundice.

BILIRUBIN

Micro-Method

When frequent serum bilirubin determinations are required as in neo-natal jaundice due to Rh⁰(D) or other incompatibility, it becomes desirable to be able to determine at least total bilirubin on amounts of blood obtainable by finger, heel, or ear puncture.

Collection of Specimen:

See precautions as in macro-procedure. Make collection as outlined under Micro-collection (p. 70) obtaining 0.3 to 0.5 ml. of whole blood.

Analysis of Serum:

1. Pipet into a small test tube (B) exactly 0.9 ml. distilled water. Add to this exactly 0.1 ml. serum.
2. Mix well and remove to another tube (A) 0.5 ml. of the diluted serum.
3. Add to tube (B) 0.5 ml. diazo blank solution and to tube (A) 0.5 ml. diazo reagent.
4. Add 1.5 ml. methyl alcohol to tubes (A) and (B), mix well and read after 15 minutes.

Calculation:

$$D \times 5 K = \text{mg. \% total bilirubin.}$$

Caution:

The use of the same value for K for both macro- and micro- modifications of the bilirubin procedure assumes that the same size cuvet is used for standardization, and for reading the unknowns.

BROMIDE
In Serum, Urine, and Spinal Fluid

References:

1. Katzenelbogen, S. and Czarski, T., *Proc. Soc. Exper. Biol. and Med.* **32**, 136 (1934).
2. Diethelm, O., *J. Nerv. and Mental Dis.* **71**, 151 (1930).
3. For a more specific and accurate procedure see: *Biochem. J.* **56**, 588 (1954).

Principle:

Bromide reacts with gold chloride to form gold bromide which gives a red color suitable for photometry. Chloride decreases the color formation. Sodium chloride is incorporated into the reagents to correct for the effect of serum and cerebrospinal fluid chloride.

Apparatus:

Wavelength 440 mμ (not critical)

Reagents:

Trichloroacetic acid - chloride solution "TCA-NaCl."

Place 0.6 g. reagent grade sodium chloride into a 100 ml. volumetric flask, dissolve in about 10 ml. distilled water. Add 10 g. crystalline trichloroacetic acid, dissolve and dilute to the mark.

Gold chloride 0.5%

Dissolve the contents of one ampoule (15 grains $\text{AuCl}_3 \cdot \text{HCl} \cdot 3\text{H}_2\text{O}$) in 195 ml. distilled water. Stir until dissolved and filter using an ashless filter paper.

Sodium bromide - Stock Standard

Place 1.000 g. reagent grade sodium bromide in a 100 ml. volumetric flask. Dissolve and dilute to the mark with distilled water.

Dilute working standard

Transfer 3.00 ml. of stock standard to a 100 ml. volumetric flask, dilute to the mark with distilled water and mix. Prepare immediately before use.

Calibration:

In a series of six test tubes, place 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 ml. of the dilute standard. Add water to make 5.0 ml. These standards represent concentrations of 0, 30, 60, 90, 120, and 150 mg./100 ml. of sample. Proceed immediately to the color development.

To each standard tube add 4.0 ml. TCA-NaCl solution, and mix. Then add 1.0 ml. 0.5% gold chloride solution and mix well.

Within 10 minutes read each solution against the blank set at $T = 100$ or $D = 0$ at

a wavelength of 440 mμ. Plot the results which should fall on a straight line. Prepare a calibration table.

Procedure :

Serum: In a tube which can be centrifuged place 8.0 ml. TCA-NaCl. Add dropwise with constant shaking 2.0 ml. serum. Shake vigorously and allow to stand 30 minutes. Centrifuge or filter through a small Whatman #2 or #44 paper.

In a cuvet (sample) place 5.0 ml. clear filtrate, 4.0 ml. distilled water and 1.0 ml. 0.5% gold chloride. Mix well.

In another cuvet (blank) place 4 ml. TCA-NaCl, 5 ml. distilled water and add 1.0 ml. 0.5% gold chloride and mix.

Within 10 minutes read sample against the blank set at T = 100 (or D = 0) at a wavelength of 440 mμ. Obtain the concentration of bromide from the calibration table.

Urine: Proceed as under blood using a dilution of well-mixed urine.

Spinal fluid: Proceed as for serum.

Interpretation:

There is normally only a very small amount of bromide in serum and some of this value is undoubtedly due to non-specific color developed by serum filtrate. The normal low value is usually said to be about 2-3 mg. per 100 ml. of serum.

Symptoms of bromide intoxication appear when the serum bromide level reaches 100 to 200 mg. % although there is considerable individual variability. Bromide partially replaces chloride in the tissue electrolyte pattern and therapy consists in replacement of bromide by chloride. This may require several days to several weeks.

BROMSULFALEIN TOLERANCE AND CLEARANCE TEST (Sulfobromophthalein)

References

1. Rosenthal, S. M., and E. C. White; J. Am. Med. Assoc. 84, 1112 (1925).
2. Gaebler, O. H.; Am. J. Clin. Path. 15, 452 (1945)
3. Dragstedt, C. A. and M. A. Mills; J. Lab. Clin. Med., 21, 1306 (1936)
4. Hamilton, R. H.; Fed. Proc. 6, 258 (1947)
5. Goodman, R. D., and Kingsley, George R.; J. A. M. A., 153, 462 (1953)

Principles

The dye, sulfobromophthalein (Bromsulfalein) is injected intravenously. It is rapidly removed from the plasma by the normal liver; in liver disease larger amounts are retained in the circulating plasma after a given time interval.

The principle of the chemical determination is based on the fact that the dye is colorless in acid and reddish-violet in alkaline solution. This property is utilized in the determination by measuring its light absorption in an alkaline medium. In the presence of bilirubin, hemolysis or lipemia, the simple method described results in errors since the absorption characteristics of the interfering substances are also altered when the pH is changed. Gaebler (ref. 2) avoids this difficulty by measuring the absorbance at 620 mμ and at 565 mμ, thus compensating for the non-specific absorbances which are about the same at these two wavelengths. Hamilton (ref. 4) measures the absorbance before and after chemical bleaching of the bromsulfalein by sodium dithionite. These last two approaches are important only when the aforementioned interferences are present. Another approach which has been used is to use acetone to precipitate the proteins and at the same time extract the dye. The color is then developed in the acetone solution.

Reagents: Sodium hydroxide 0.1 N
Hydrochloric acid 0.1 N

Procedure:

Collection of specimens: The patient must be fasting (at least 10 hours post-prandial) and non-icteric, and kept in a supine position in bed for the duration of the test. The patient's weight must be known and converted to kilograms (lbs. /2.205).

2. Draw into a sterile syringe an exact amount of sulfobromophthalein so that the injected dose will be 5 mg. per kilogram of body weight. This volume should be exact to the 0.1 ml.

3. Then inject the dye into an arm vein over a period of 30 seconds.

4. Start timing the test when half of the dose has been given.

5. Five minutes later, from a vein of the opposite arm, withdraw into another syringe 5-10 ml. of blood for serum. Take further samples at appropriate times, for example at 30, 45 and at 60 minutes. For the determination of bromsulfalein clearance, take samples at about 5, 10, 15 and 20 minutes and carefully note the exact times.

Estimation of Bromsulfalein

Add 0.5 ml. of clear, unhemolyzed serum to 6.0 ml. of 0.1 N NaOH in a photometer. Mix well. Read against a blank prepared in the same way but using 6.0 ml. of 0.1 N HCl. Set the blank at $T = 100$ ($D = 0$) using a wavelength of 575 mμ.

Read the retention in per cent from the calibration table which is based on the administration of 5 mg. per kilogram of body weight. (If the dose was 2 mg. per kilogram, multiply by 2.5 to obtain per cent retention.)

Standardization

Since the results of the test are expressed in terms of per cent retention of the dye, it is obvious that we must determine or assume the "volume of distribution" into which the dye is diluted.

The calculation is commonly based on the following assumptions:

- (1) 10% of the body weight is circulating blood.
- (2) 50% by volume of the blood is plasma.
- (3) Therefore 5% of the body weight is plasma or 50 ml. per kilogram of body weight is plasma.

Since 5 mg. per kilogram of body weight is injected this is 5 mg. per 50 ml. plasma or 10 mg. per 100 ml. of plasma. 10 mg. % is considered to be the initial concentration of dye in the plasma or 100% retention.

Bromsulfalein Standard Solution

Pipette carefully and accurately, 0.1 ml. of 5% bromsulfalein solution into a 500 ml. volumetric flask and dilute to volume with approximately 0.1 N NaOH. Mix well by inversion.

Calibration Standards

Into a series of photometer cuvetts, transfer 0.0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 ml. of bromsulphalein standard solution. Make each tube up to 6.0 ml. with 0.1 N NaOH. Mix well, then add 0.5 ml. of normal pooled serum, and mix well again. These tubes represent 0, 10, 20, 40, 60, 80 and 100% retention of the dye using the assumptions given above. Using the 0.0 tube as a blank $T = 100$ ($D = 0$) record the transmittance of each tube and plot the optical density vs. % retention and mg.% as illustrated on p. 54a. Prepare a calibration graph as outlined on p. 55.

NOTES

In this simple analysis the serum must be clear, with no hemolysis, bilirubinemia, or lipemia. If it is wished to avoid the effects of such interferences it is necessary to proceed with one of the modifications referred to above (ref. 2 or 4) or to use the following extraction procedure.

1. Into a 15 ml. centrifuge tube place about 2 ml. acetone. Add 0.5 ml. of serum, dropwise with agitation. Mix well. Heat to boiling in a boiling water bath. Cool. Centrifuge.

2. Decant the supernatant fluid into a tube graduated at 7 ml. Add about 1 ml. of acetone to the residue in the centrifuge tube, mix well with a wooden applicator stick, allow to stand 2 minutes and centrifuge. Decant the supernatant fluid into the 7 ml. graduated tube. Repeat once more.

3. Add distilled water to about 7 ml. Mix well. Then add 2 ml. ether and mix by inversion. Allow to stand 3-5 minutes to separate the phases. Remove the ether phase by suction. Repeat once more.

4. Adjust the acetone water layer to 10.0 ml. with 0.1 N NaOH and mix. Read against a blank of 3 ml. of acetone plus 3 ml. of water diluted to 10.0 ml. with 0.1 N NaOH.

Calculation:

Multiply the chart reading by 1.54 (10.0/6.5) to obtain % retention or mg.%.

Interpretation

Normal individuals after injection of a 5 mg. per kilogram of body weight dose have a plasma retention of less than 10% at 30 minutes, <7% at 45 minutes and usually 0-1% at 60 minutes.

If a 2 mg. per kilogram body weight dose is used as originally proposed by Rosenthal and White (ref. 1) and as readvocate by Lorber and Shay, *Gastroenterology* 20, 262 (1952), to minimize entero-hepatic recirculation of the dye, the 30 minute retention will be less than 4%.

The rate of removal of bromsulfalein by the normal liver is determined by (1) the rate of blood flow through the liver, (2) the functional capacity of the polygonal cells of the liver, and (3) the degree of patency of the bile duct system. In obstructive jaundice, retention is very high, out of proportion to the actual damage to the functional capacity of the liver. Zieve et al., *J. Lab. Clin. Med.* 37, 40 (1951), have devised a correction to be applied when the one-minute bilirubin of Watson is elevated beyond normal limits. When corrected in this way, the test more closely estimates the functional capacity of the polygonal cells even in obstruction. The diversion of blood from the usual liver channels into collateral vessels is an important cause of high retention not only in cirrhosis but also in viral or chemical hepatitis and also in heart failure.

It is doubtful that the bromsulfalein test adds much to the diagnosis or prognosis of liver disease in the presence of jaundice.

Bromsulfalein Clearance

In order to express more clearly the rate of disappearance of the dye, the concept of liver clearance (analogous to renal clearance) has been introduced and applied to not only this dye but to other substances such as galactose and hippuric acid. This approach necessarily applies to substances not normally found in serum, since the only way to measure the clearance is by the rate of decrease in serum concentration. Goodman and Kingsley (ref. 5) have used this method quite extensively and the description below is based on their work.

Procedure

Collection of samples

1. Injection of dye and the collection of samples is carried out as indicated under the tolerance test with the injection and collection very carefully timed (mid-point time of injection and collection). The collection of samples should be completed by the end of 20 minutes.

Interpretation

Normal individuals after injection of 2 mg. per kilogram of body weight have a plasma retention of less than 10% at 30 minutes, 47% at 45 minutes and usually 8-12% at 60 minutes.

If a 2 mg. per kilogram body weight dose is used as originally proposed by Rosenthal and White (1957) and as recommended by Lachor and others (Gastroenterology 35, 1961) (1962) to minimize splanchnic retention of the dye, the 30 minute retention will be less than 4%.

The rate of removal of bromothalein by the normal liver is determined by (1) the rate of blood flow through the liver, (2) the functional capacity of the polygonal cells of the liver, and (3) the degree of patency of the biliary tract system. In obstructive jaundice retention is very high, and of proportion to the degree of damage to the functional capacity of the liver. Kline et al., J. Lab. Clin. Med. 57, 46 (1961) have devised a correction to be applied when the one-minute bilirubin of Watson is elevated beyond normal limits. When corrected in this way, the test more closely estimates the functional capacity of the polygonal cells even in obstruction. The diversion of blood from the renal liver sinusoids into collateral vessels is an important cause of high retention not only in cirrhosis but also in viral or chemical hepatitis and also in heart failure.

It is doubtful that the bromothalein test adds much to the diagnosis or prognosis of liver disease in the presence of jaundice.

Bromothalein Clearance

In order to express more clearly the rate of disappearance of the dye, the concept of liver clearance (analogous to renal clearance) has been introduced and applied to not only this dye but to other substances such as glucose and inorganic acids. This approach necessarily applies to substances not normally found in water, since the only way to measure the clearance is by the rate of excretion in serum concentration. Goodwin and Kirby (1961) have used this method quite extensively and the description below is based on their work.

Procedure

Collection of samples

1. Injection of dye and the collection of samples is carried out as indicated under the tolerance test with the injection and collection very carefully timed (half-point time of injection and collection). The collection of samples should be completed by the end of 30 minutes.

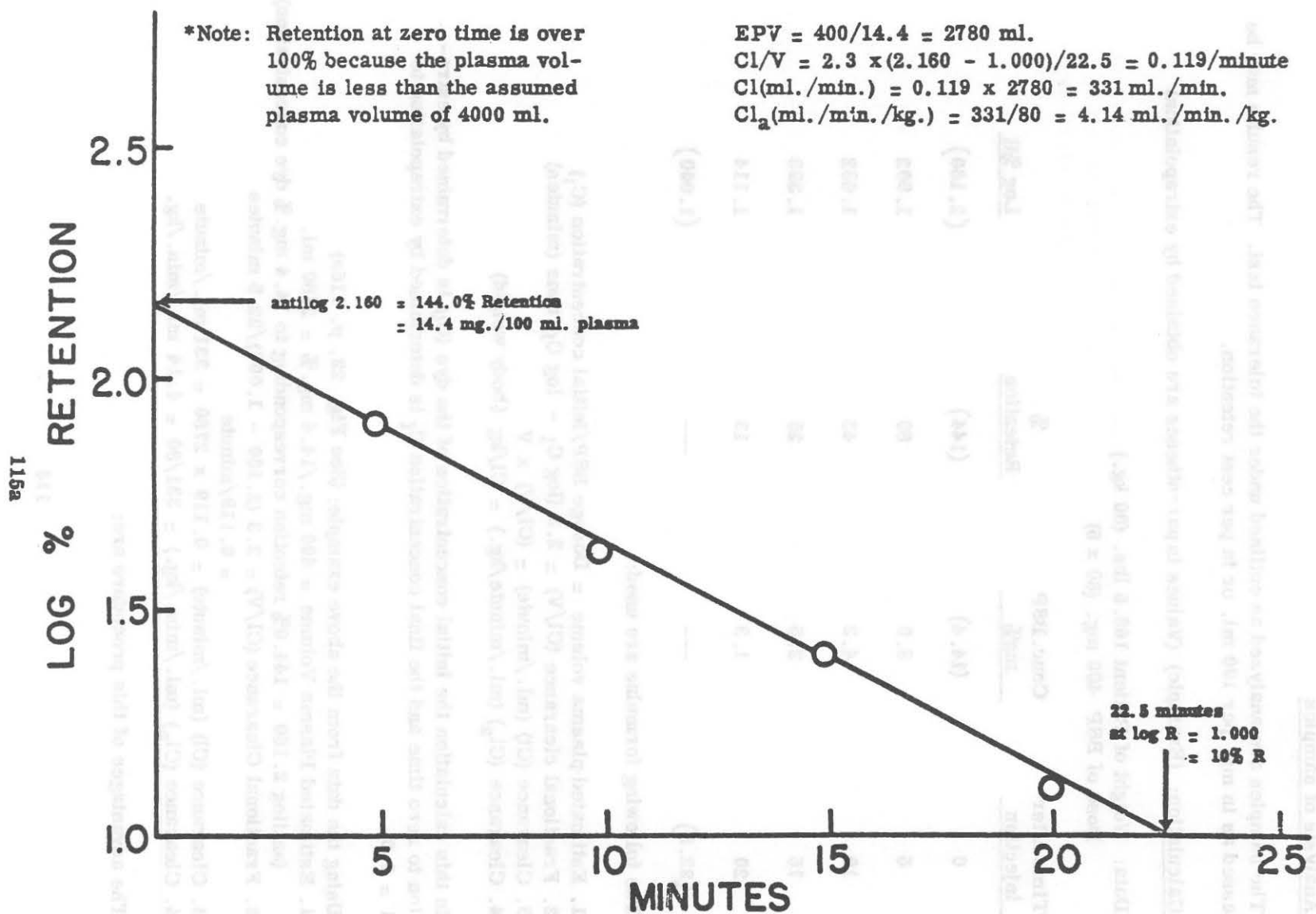


Fig. 22. Bromsulfalein clearance.

Analysis of samples

The samples are analyzed as outlined under the tolerance test. The results may be expressed as in mg. per 100 ml. or in per cent retention.

Calculation: (Example) (Values in parentheses are obtained by extrapolation)

Data: Weight of Patient 168.5 lbs. (80 kg.)

Dose of BSP 400 mg. (80 x 5)

<u>Time after injection</u>	<u>Conc. BSP mg%</u>	<u>% Retention</u>	<u>Log %R</u>
0	(14.4)	(144)	(2.160)
5	8.0	80	1.902
10	4.3	43	1.632
15	2.5	25	1.398
20	1.3	13	1.114
(22.5)	---	---	(1.000)

The following formulae are used:

1. Estimated plasma volume = Dosage BSP/Initial concentration (C_i)
2. Fractional clearance (Cl/V) = $2.3 (\log C_i - \log C_f)$ time (minutes)
3. Clearance (Cl) (ml./minute) = (Cl/V) x V
4. Clearance (Cl_2) (ml./minute/kg.) = Cl/kg (body weight)

In this calculation the initial concentration of the dye (C_i) is determined by extrapolation to zero time and the final concentration C_f is determined by extrapolation to $\log R = 1.0$.

Using the data from the above example: (See Fig. 22, p. 115a)

1. Estimated Plasma Volume = $400 \text{ mg.} / 14.4 \text{ mg. \%} = 2780 \text{ ml.}$
(antilog 2.160 = 144.0% retention corresponding to 14.4 mg. % dye concentration)
2. Fractional Clearance (Cl/V) = $2.3 (2.160 - 1.000) / 22.5 \text{ minutes}$
= 0.119/minute
3. Clearance (Cl) (ml./minute) = $0.119 \times 2780 = 331 \text{ ml. /minute}$
4. Clearance (Cl_2) (ml./min./kg.) = $331 / 80 = 4.14 \text{ ml. /min. /kg.}$

The advantages of this procedure are:

1. The accuracy and precision are both increased by collection and analysis of a series (rather than one) of blood specimens and by the method of calculation.

2. The method gives an estimate of the plasma volume as well as a numerical single value expressing a rate of clearance of the dye.

3. The test is completed in 20 minutes.

4. The clearance results do not depend upon the accuracy of measurement of the injected dye (although the estimate of plasma volume does).

Interpretation:

Clearance

Normals (28)	5.33 \pm 0.75 ml. /min. /kg.	None below 4 ml. /min. /kg.
Cirrhosis (18)	2.06 \pm 0.30 "	None above 3 "
Alcoholics (35)	4.45 \pm 0.63 "	37% of the values were lower than lowest of the control group

Plasma Volume

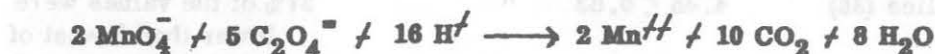
Normals	40.9 \pm 6.16 ml. /kg.
Cirrhosis	50.6 \pm 6.0 "
Alcoholics	38.4 \pm 4.93 "

CALCIUM
Serum, Urine, etc.

References:

- (1) Clark, E. P. and J. B. Collip: *J. Biol. Chem.*, **63**, 461 (1925)
- (2) deLoureiro, J. A. and G. F. Janz: *Biochem. J.*, **38**, 16 (1944)
- (3) Rehell, B.: *Scand. J. Clin. Lab. Invest.*, **6**, 335 (1955)
- (4) Patton, J. and Reeder, W.: *Anal. Chem.*, **28**, 1026 (1956)
- (5) Nordin, E. C. and R. Fraser: *Lancet*, **270**, 826, 2 June (1956)

Principle: Calcium is precipitated as the oxalate, which is washed free of excess oxalate and then titrated in hot acid against KMnO_4 .



Apparatus: Micro-buret 5 ml. in 0.01 ml. divisions.

Reagents:

1. Ammonium hydroxide 0.35 N. Dilute 2 ml. concentrated NH_4OH to 100 ml. with distilled water.
2. Ammonium hydroxide 4 N. Dilute 22 ml. concentrated NH_4OH to 100 ml. with distilled water.
3. Ammonium oxalate 4% w/v in water.
4. KMnO_4 -- 0.1 N stock. Dissolve 3.3 g. pure reagent grade KMnO_4 crystals in 1000 ml. distilled water. Bring to a boil. Cover with a beaker and allow to cool and stand in the dark for two to three days or longer before using.
5. KMnO_4 -- dilute working solution. Dilute 10 ml. of stock 0.1 N solution to 100 ml. with water. Prepare and standardize just before use.
6. Sodium oxalate stock 0.1 N. Weigh out exactly 6.7010 g. of pure dry reagent grade salt, dissolve and dilute to 1 liter with 1 N H_2SO_4 . This solution is stable for several months.
7. Sodium oxalate working standard. Dilute 10.00 ml. 0.1 N stock solution to 100 ml. with 1 N H_2SO_4 . Mix well. Prepare weekly.

8. Sulfuric acid 1 N. Dilute 3 ml. concentrated H_2SO_4 to 100 ml. with distilled water. Mix well.
9. Phenol red - approximately 0.04% in water.
10. Acetic acid 10%. Dilute 10 ml. of glacial acetic acid to 100 ml. with distilled water.

PRECIPITATION

Serum:

1. Clean a 12 ml. heavy walled conical centrifuge tube by filling with dichromate cleaning solution and allowed to stand for at least 10 minutes, preferably overnight. Rinse thoroughly with distilled water and drain.
2. Add 2.00 ml. cell-free serum to the tube using an Ostwald pipet.
3. Add 2 ml. distilled water.
4. Add 1.0 ml. ammonium oxalate 4% solution.
5. Mix well by tapping and allow to stand for one hour.
6. Centrifuge at highest speed for 10 minutes (do not use an inclined head), invert quickly and drain for 5 minutes, inverted on a piece of clean filter paper; then dry the mouth of the tube with a piece of filter paper.
7. Blow into the tube about 1.5 ml. of 0.35 N NH_4OH breaking up the precipitate. Rinse the sides of the tube with 1.5 ml. more of the solution.
8. Recentrifuge, invert quickly and drain 5 minutes, drying the mouth of the tube after draining, as before.
9. Repeat washing, centrifugation and draining.

Urine:

1. Pipet exactly 1-5 ml. (depending on the expected calcium content) of urine into a conical tube.
2. Add 1 drop of phenol red indicator and then 4 N NH_4OH drop by drop until the indicator is pink.
3. Immediately add 10% acetic acid drop by drop until the indicator just turns yellow.

4. Add 1 ml. of ammonium oxalate 4%.
5. Mix well and let stand 1-2 hours at room temperature.
6. Proceed with centrifuging and washing as in serum calcium but wash the precipitate two extra times.

Food, Tissue and Feces:

1. Ash an appropriate dried aliquot of sample at 600°C., for several hours, to a white ash. If a dark ash is obtained, add a few drops of concentrated HCl, dry and reignite at 600°C.
2. Dissolve the ash in a drop or two of concentrated HCl.
3. Transfer quantitatively and bring to volume in a 50 ml. volumetric flask.
4. Proceed with precipitation and washing as in the urine calcium procedure, using a 5.00 ml. aliquot.

TITRATION OF STANDARD

Into a 12 ml. centrifuge tube pipet exactly 2.00 ml. 0.01 N sodium oxalate solution, using an Ostwald pipet. Heat for two minutes in a boiling water bath and titrate while still hot (keep in a beaker of hot water) with 0.01 N (approximately) KMnO_4 solution to the first permanent pink; titrate slowly at first, allowing each drop to react before adding the next.

$$\text{Correction factor} = 2 \text{ ml. / ml. } \text{KMnO}_4 \text{ used}$$

The factor should be between 0.95 and 1.05. If it is not, adjust the KMnO_4 solution by addition of more water or more KMnO_4 .

TITRATION OF UNKNOWN

1. Add 2.0 ml. 1 N H_2SO_4 , tap gently to break up the precipitate.
2. Heat for 2 minutes in the boiling water bath, until all of the precipitate is dissolved.
3. Titrate, using the precautions noted above, to the first permanent pink.

CALCULATIONS

One (1) ml. of 0.01 N sodium oxalate solution (or of 0.01 N KMnO_4) is equivalent to 0.2 mg. of calcium.

$$1.00 \text{ ml.} \times 0.01 \text{ N} = 0.01 \text{ mEq.}$$

$$\frac{0.01 \text{ mEq.} \times 40 \text{ (at. wt. Ca)}}{2 \text{ (valence of Ca)}} = 0.2 \text{ mg. Ca.}$$

$0.2 \times \text{ml. permanganate (0.01 N)} \times 100/2 = \text{mg. Ca per 100 ml. serum}$
or $10 \times (\text{ml. permanganate}) \times (\text{correction factor}) = \text{mg\% Ca.}$

Example: 2.00 ml. 0.01 N sodium oxalate required 1.980 ml. of approximately 0.01 N KMnO_4 for titration.

$$\text{Factor} = 2.00/1.98 = 1.01$$

The calcium oxalate precipitated from 2.00 ml. of serum, titrated as above, required 1.060 ml. of this permanganate for titration.

$$10 \times 1.060 \times 1.01 = 10.7 \text{ mg. Ca per 100 ml. serum}$$

Urine and Other Samples:

The calculations are very similar, except that the degree of dilution and size of the sample must be considered.

Notes:

1. Extreme care is required in mixing, washing and draining, to avoid loss of some of the precipitated calcium oxalate.
2. The solution, during the titration, must be kept between 70 and 75°C. to insure a stoichiometric reaction. A brown color, (MnO_2) obtained by titration at lower temperatures, or by too rapid initial titration, will result in inaccurately high values.
3. When the first drop of KMnO_4 is added the pink color persists for some time. As soon as catalytic amounts of manganous (Mn^{++}) salts are formed (see reaction p. 118) the reaction proceeds more rapidly.
4. In the analysis of urine it is advisable to use fresh samples if at all possible. For 24-hour collections and for brief preservation add 4 ml. glacial acetic acid per 100 ml. of urine. Measure the total volume before aliquotting. The urine calcium determination is less specific due to organic substances being adsorbed to the precipitated calcium oxalate (such as murexide).

5. Other methods which might be used include:

- (a) Oxalate precipitation methods using permanganate with added MnSO_4 to catalyze the reaction at room temperature (Ref. 2) with subsequent analysis by titration iodimetry or photometry.
- (b) Chelatometric methods based on the formation of an extremely stable soluble calcium complex with ethylene-diamine-tetraacetic acid (EDTA) together with the use of specific indicators (Ref. 3 and 4).

INTERPRETATION

Serum Calcium:

All of the calcium in the blood is confined to the plasma. The normal range for serum total calcium is between 9 and 11 mg. per 100 ml. serum. (5 mEq. per liter; 2.5 mM. per liter). In infants (especially premature) with adequate Vitamin D intake the level may be as high as 12 to 13 mg. %.

About 1/3 of the calcium of the serum is bound to protein and is "inactive" physiologically. The remainder is composed of ionized (mostly) and unionized (citrate?) calcium, only the ionized portion is involved in the control of serum calcium level and in muscle irritability. The level of total serum calcium is affected by:

- (1) Deficient intestinal calcium absorption.
- (2) Alteration in parathyroid hormone secretion.
- (3) Changes in serum inorganic phosphate.
- (4) Alteration in plasma proteins.

A recent formula (Ref. 5) for the proportion bound to protein is:

$$\text{Protein-bound Calcium} = (7.6 \times \text{albumin}) \div (2.9 \times \text{globulin}) - 3.1$$

in which protein-bound calcium is expressed in per cent of total calcium and albumin and globulin are expressed in g. per 100 ml. serum. For example:

A serum gave the following values —	Total calcium	10.8 mg. %
	Albumin	4.6 g. %
	Globulin	2.1 %

$$\begin{aligned}\text{Protein Bd. Ca} &= (7.6 \times 4.6) \div (2.9 \times 2.1) - 3.1 \\ &= 35 \div 6.1 - 3.1 \\ &= 38\%\end{aligned}$$

$$\begin{aligned}10.8 \times 0.38 &= 4.1 \text{ mg. \% calcium is protein-bound} \\ &6.7 \text{ mg. \% calcium is diffusible.}\end{aligned}$$

Decreases in Serum Calcium are seen in:

With Values given as:

Hypoparathyroidism	Less than 6 mg. %
Rickets (Vitamin D deficiency) (children)	Usually 8 - 9.5 mg. %
Steatorrhea (as in sprue, coeliac disease)	Usually 8 - normal
Renal failure (secondary to elevated $\text{PO}_4^{=}$)	As low as 6 mg. %

Tetany may be seen in patients with decreased total serum calcium with abnormal reflexes below 8 mg. % and carpopedal spasm below 7 mg. %. These values are modified to some extent by variation in serum protein level and acid-base balance in so far as these modify the proportion of the total calcium present as ionized Ca^{++} , since it is the level of ionized calcium which influences the irritability of muscle. The overall irritability of muscle is also influenced by many other factors as well.

A decrease in serum calcium is never the cause of excessive bleeding. The serum calcium level required for coagulation is so small that it is always present in excess. In massive transfusions, citrate may bind enough Ca^{++} to affect coagulability if combined with other effects. Oxalate and citrate both may cause tetany by binding Ca^{++} .

Increases in Serum Calcium are seen in:**Representative Values:**

Hyperparathyroidism	12 - 20 mg. %
Hypervitaminosis D	12 - 17 mg. %
Multiple myeloma	12 - 17 mg. %

In most other diseases, including bone disease, the serum calcium is maintained within normal limits due to very close physiological control and the large reserve storehouse of readily available calcium -- the bones.

Calcium Excretion:

On the ordinary diet containing 0.7 - 0.9 g. of calcium daily, about 75% of the calcium appears in the feces, the remainder appearing in the urine. The proportion depends somewhat on calcium binding substances present in the diet.

There is an increased urinary excretion of calcium in hyperparathyroidism and in multiple myeloma. A decreased urinary excretion of calcium is seen in rickets due to impaired intestinal absorption of calcium.

Calcium Balance Tests:

In some disease states the net loss of calcium from the body may be so slow (over a period of 20 to 30 years in the case of osteoporosis) that the serum calcium values or the urinary excretion rate may not seem to be affected even though the excretion is greater than the intake. In these cases a Calcium Balance may be carried out as follows:

The patient is placed on a low calcium diet containing about 100 - 150 mg. of calcium and 500 - 700 mg. of phosphorus daily, and three days are allowed for the patient to become adjusted to the diet. A feces marker (carbon, carmine, or gentian violet) is given before breakfast the day collections are started. Three to four day collections are made at the end of which another marker is given before breakfast at which time urine collection ceases for that period. Feces collection is made from marker to marker, including in the test the specimen containing the first marker but excluding that containing the second marker.

The urine and fecal calcium is determined and the average daily excretion compared to the known intake.

Calcium Infusion Tests: (Ref. 5)

In order to avoid the long tedious procedures involved in calcium balance studies, the rate of excretion of calcium in the urine after infusion of 400 - 1200 mg. of calcium as the gluconate (or other non-irritant salt of calcium) has been studied and has served as an index of therapeutic effectiveness in osteoporosis, hypoparathyroidism and several other diseases. (Perloff, W.H., J.H. Boutwell, Jr., and R.H. Maas: J. Amer. Ger. Society, 4, 760 (1956). (Shilling, A., and Lazlo, D., Rate of urinary calcium excretion following intravenous infusion. Proc. Soc. Exper. Biol. & Med., 78, 286 1951.)

CALCIUM BALANCE DIET (MODIFIED AUB DIET)

Place patient on a diet containing 100 mg. of calcium per day for 6 days, collect 24-hour samples of urine for the last 3 days of the diet period. Pool and analyze for calcium.

An excretion of 300 mg. or less of calcium for the 3-day period is considered normal. In hyperparathyroidism, considerably over 300 mg. will be excreted.

SAMPLE DIET

	<u>Allow</u>	<u>Avoid</u>
Soup	None	Omit entirely
Meat, Fish, Poultry	2 of following daily	Use no other meat, fish or poultry
	60 grams lean beef	
	50 " chicken	
	50 " lamb	
	50 " mackerel	
	65 " lean veal	
	80 " turkey	
	70 " halibut	
	60 " codfish	

Cereal, Eggs and Milk Omit entirely

Vegetables	3 of following daily	Use no other vegetables
0.009 gram calcium	40 grams peas	
	45 " asparagus	
	65 " summer squash	
	60 " winter squash	
	75 " potatoes	
	80 " fresh tomatoes	
	90 " cucumbers	
	100 " corn	
	100 " egg plant	
	130 " fresh tomato juice	

Potato Substitute	1 of following daily	Avoid all other
0.003 grams calcium	25 grams dry rice	
	15 " spaghetti	
	15 " macaroni	
	30 " hominy	

CALCIUM BALANCE DIET (MODIFIED AUB DIET) (Cont.)

	<u>Allow</u>	<u>Avoid</u>
Fruits	4 of following daily	Avoid all other
0.007 grams calcium	50 grams cantalope	
	50 " cherries	
	50 " grapefruit	
	50 " plums	
	50 " pineapple	
	50 " apricots	
	60 " pears	
	80 " grapefruit juice	
	90 " peaches	
	100 " bananas	
	100 " apples	
	100 " watermelon	
	100 " fresh tomato juice	

Beverages	Fluid intake should be constant throughout test period.	Avoid all other
	1 cup coffee at breakfast	
	1 cup tea at noon & evening meal	
	Water may be had between meals	

Bread	30 grams bread each meal	Avoid all other foods
0.008 grams calcium		

Miscellaneous	Sugar as desired
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CALCIUM Serum

Reference:

Ferro, P.V., and Ham, A.B., *Am. J. Clin. Path.*, **28**, 208 (1957).

Principle:

Calcium is precipitated as the insoluble salt of chloranilic acid. It is washed free of excess chloranilic acid, using isopropyl alcohol. The precipitate of calcium chloranilate is then dissolved in Na_4EDTA (ethylenediamine tetra-acetic acid, tetra sodium salt). The resulting pink solution is compared photometrically against similarly prepared standards and blanks.

Reagents:

1. Chloranilic acid (1%). Dissolve 1 g. of chloranilic acid in 50 ml. of distilled water containing 7 ml. of 1 N NaOH. Mix and dilute to 100 ml. with distilled water.
2. Na_4EDTA (5%) (ethylenediaminetetraacetic acid, tetra-sodium salt). Dissolve five grams of Na_4EDTA in 100 ml. distilled water.
3. Isopropyl alcohol 50%. Mix equal volumes of isopropyl alcohol and distilled water.
4. Calcium standard. Weigh out exactly 0.2497 g. reagent grade calcium carbonate (CaCO_3). Wash into a 1 liter volumetric flask. Add gradually, 10 ml. of 1 N HCl, using it to rinse down the sides of the flask. When solution is complete, dilute to volume and mix. 1 ml. = 0.1 mg. calcium.

Procedure:

Into four 12 ml. heavy duty, conical, pyrex centrifuge tubes cleaned as outlined on p. 119, add reagents and proceed as outlined.

	<u>Blank</u> (Ml. to be added)	<u>Std₁</u>	<u>Std₂</u>	<u>Unkn.</u>
H_2O	2	1	---	---
Standard Calcium	---	1.00	2.00	---
Serum	---	---	---	2.00
Chloranilic acid	1	1	1	1

2. Mix the contents of each tube well and allow to stand at room temperature for at least 30 minutes.
3. Centrifuge, decant and drain on a filter paper for 5 minutes. Wipe the lip of the tube free of the last drop.
4. Blow in 4 ml. of 50% isopropyl alcohol so as to break up the precipitate. Mix.
5. Centrifuge, decant, and drain as in step 3.
6. Add 1 drop of distilled water to each tube. After standing one minute gently tap the tube to break up the precipitate.

7. Add 4.00 ml. of Na_4EDTA to each tube, mix well and after complete clearing (about 5 minutes) compare photometrically at 520 m μ against the blank set at 100% T.

Calculations:

$$\text{Ca (mg. \%)} = (D_u/D_s) \times C_s \times (100/2)$$

$$\text{(For Std}_1\text{)} = (D_u/D_s) \times 0.1 \times (100/2)$$

$$= (D_u/D_s) \times 5$$

$$\text{(For Std}_2\text{)} = (D_u/D_s) \times 10$$

Interpretation:

See previous method.

CARBON DIOXIDE OF PLASMA CAPACITY AND CONTENT

Reference:

Van Slyke, D. D., and Cullen, G. E., J. Biol. Chem., **30**, 289 (1917)

Principle:

The carbon dioxide capacity is determined after equilibrating plasma in a 300 ml. separatory funnel filled with an alkali mixture whose carbon dioxide tension approximates that of normal arterial blood. By this technique the sample of plasma combines with as much CO_2 as it is able to hold under normal CO_2 tension in the circulating plasma (40-45 mm. Hg.).

The carbon dioxide content determination requires special care in plasma collection and preservation to insure that the content of gases does not change until the analysis is completed. In this determination we attempt at all times to keep the blood at the CO_2 tension that existed in the body. The blood should be collected without exposure to air and transferred immediately to a tube under mineral oil. For details read further under methods of collection and preservation of specimens. If blood is collected in a "Vacutainer" tube, mineral oil is not necessary and this technique requires centrifuging without removing the stopper, then rapid removal and measurement of the sample. The tube is then restoppered immediately, thus preserving the sample for a second determination or a pH determination if it is also desired.

ACID-BASE BALANCE - DISCUSSION

Reference:

Levinson and MacFate: pp. 176-181

Peters and Van Slyke: Vol. I, Chapter 18

There are three buffer pairs in the blood: (1) the primary-secondary phosphates; ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$) which have little clinical significance in maintaining a constant pH in blood; (2) acidic-basic proteins, particularly hemoglobin, which act primarily to prevent carbon dioxide from changing the pH of venous plasma more than about 0.03 pH units from that of arterial plasma, and to aid in the process of transportation and liberation of carbon dioxide; and (3) carbonic acid-bicarbonate ($\text{NaHCO}_3/\text{H}_2\text{CO}_3$) which is the chief buffer against strong acids or bases.

The hydrogen ion concentration of the blood is directly related to the ratio of bicarbonate to dissolved carbon dioxide concentration, by the equation:

$$\text{pH} = \text{pK}_a + \log \frac{(\text{NaHCO}_3)}{(\text{H}_2\text{CO}_3)}$$

This is the "buffer equation" of Henderson and Hasselbalch.

Therefore the hydrogen ion concentration (acidity) of the blood increases if the denominator of this fraction is increased, or if the numerator is decreased. The acidity

decreases if the denominator decreases or if the numerator increases. The body mechanisms (lungs, kidneys) attempt always to maintain a constant hydrogen ion concentration by making the numerator and denominator vary together, thus keeping a relatively constant quotient or ratio. When a strong acid, such as B-hydroxybutyric acid or acetic acid, is introduced into the blood, it reacts with the bicarbonate present to form the sodium salt of the stronger acid, plus carbon dioxide. Then enough of the latter is excreted by the lungs to restore the above ratio nearly to normal. Thus the pH of the plasma is maintained relatively constant, though the concentrations of CO_2 and bicarbonate in it are decreased.

Conditions which upset the ratio of H_2CO_3 to HCO_3^- can be grouped under four heads:

1. Primary alkali (bicarbonate) excess. This condition is known as "alkalosis." It may be caused by the ingestion of large amounts of bicarbonates or other alkalies, or by the loss of HCl from the stomach in extensive vomiting, as in pyloric obstruction or in toxemias of pregnancy. This acid is formed by the stomach from NaCl, leaving NaHCO_3 in the blood. If then the HCl is not reabsorbed, but is vomited, there will come to be an excess of bicarbonate in the blood.
2. Primary alkali deficit (known as "acidosis"). It is found particularly in diabetes mellitus and in terminal nephritis; also in severe diarrheas of infants, in starvation, and in ether or chloroform anesthesia. It is due either to loss of NaHCO_3 , or to formation of sodium salts of non-volatile acids, which are not available for buffer action. These are excreted by the kidneys, which attempt to conserve some of the sodium by formation of ammonia from glutamine (and other amino acids) to replace sodium. Excretion of salts is accompanied by loss of fluids and dehydration. Therapy: Administer physiological saline, glucose and insulin (in diabetes) to prevent formation of acids. Bicarbonate administration is usually not necessary and may be harmful if carried too far.
3. Primary CO_2 excess. Rare; occurs in morphine poisoning and in Ayerza's disease - fibrosis of the lungs.
4. Primary CO_2 deficit. Caused by over-ventilation of lungs. Rarely this is of clinical significance. It occurs in O_2 deficit, fevers, encephalitis with affection of respiratory center; and in hysteria.

The bicarbonate content of blood plasma is known as its "alkali reserve." It is easier to determine total CO_2 obtainable by mixing plasma with acid; so alkali reserve is expressed as the number of ml. of CO_2 (at 0°C . and 760 mm. Hg.) that can be obtained from 100 ml. of acidified plasma. 55 to 75 ml. per 100 ml. of plasma or 55 to 75 volumes per cent is normal. Normal pH of plasma is 7.3 to 7.5. The pH seldom goes below 7.3 (acidosis remains "compensated") until the alkali reserve gets down below 40 volumes per cent. Hypernea appears when the value falls to half of normal (moderately severe acidosis). Below 30 volumes per cent, acidosis is severe, though recovery may occur with values as low as 10 to 15 per cent. Such cases however, usually terminate in death.

A. VOLUMETRIC PROCEDURE

Principle:

Of the CO_2 that may be obtained from blood serum the greater part is present in

the form of the bicarbonate ion (HCO_3^-). Before this potential carbon dioxide can be released for measurement, it must be freed from combination. Lactic acid, a stronger acid than carbonic, is used to convert the bicarbonate ion to carbonic acid and the gas (CO_2) is extracted under vacuum. Complete removal is not possible with one extraction but if the amount of solution and the ratio of liquid phase to gas phase is kept constant, the residue of CO_2 remaining in solution can be calculated from the solubility coefficient and the temperature and pressure. At the same time correction is made for the dissolved oxygen and nitrogen that are released by the extraction. The actual volume of CO_2 extracted can be obtained by absorbing the CO_2 with alkali such as NaOH .

Apparatus:

Van Slyke Cullen Volumetric Carbon Dioxide Apparatus. See Fig. 23. p. 132a.

Reagents:

1. Lactic acid 0.1 N (approx.). Dissolve 1 ml. of lactic acid sp. gr. 1.20 in enough water to make 100 ml. of solution. Add enough phenol red indicator solution to color.
2. 1 N NaOH . To 5.5 ml. of 18 N NaOH add with mixing enough distilled water to make 100 ml. of solution.
3. Caprylic alcohol (Methyl n-hexyl carbinol) in dropping bottle.
4. Mercury in dropping bottle.

Procedure:

Note: You should become acquainted with the position of stopcocks and leveling bulb and also the feel of the stopcocks in order to control correctly the flow of mercury and solution in the apparatus before starting the determination.

1. The entire apparatus, including the capillaries above the upper stopcock E, is filled with mercury.
2. Add one drop of caprylic alcohol to cup; open the lower stopcock, and by controlling the upper stopcock, allow the caprylic alcohol to run down into the capillary above the upper stopcock.
3. Add a few drops of mercury over the caprylic alcohol in the capillary, and seal stopcock with mercury, allowing the alcohol to go into the pipet.
4. Two ml. of lactic acid is placed in the cup B (an approximate measurement; use marking on the cup).
5. One ml. of serum (measured with a pipet calibrated between marks) is run into the cup under the lactic acid without mixing, or rubber-tipped pipet is used.
6. With the leveling bulb in low position (2), open stopcock E and control the delivery of serum and lactic acid by means of stopcock F. The lactic acid is run in until the mercury reaches the 2.5 ml. mark on pipet. Close both stopcocks and remove excess lactic acid from cup. Seal with mercury.
7. With the leveling bulb lowered to very low position 3, open the lower stopcock and draw the level of the mercury (not the aqueous mixture) to the 50 ml. mark which is just above the lower stopcock.

8. Place leveling bulb securely in position 2 and remove apparatus from stand. Shake, without inverting, for two minutes to liberate the gas. Place the apparatus back in stand carefully and allow to drain for 30 seconds.

9. Draw aqueous layer (but no gas) as completely as possible into chamber D by opening lower stopcock and lowering leveling bulb. It is better to control the flow of fluid by means of the lower stopcock.

10. Close lower stopcock and place leveling bulb in position 2.

11. Open the lower stopcock to the side arm C and allow mercury to rise slowly in the apparatus. Raise leveling bulb and place at such a height that the surface of the mercury is at exactly the same level as the mercury in the apparatus. The small amount of aqueous fluid can usually be neglected. If more than 2 or 3 cm. of solution is above the mercury, the height of the mercury in the leveling bulb should be raised 1/13th of the height of the aqueous column above the inside mercury level. Close lower stopcock and place the leveling bulb back in position 2. Read the volume of gas (at the aqueous meniscus - not at the mercury level); call the volume V; record the temperature of the environment and barometric pressure at the time of determination.

When CO_2 in plasma or serum is determined, the reading of the total gas volume may be taken as the finish of the determination. A certain amount of air carried into the apparatus dissolved in the plasma and in the 0.1 N lactic acid is mixed with the CO_2 , but the correction necessary for this may be calculated from the solubility of air in water at room temperature, and in plasma at 38°C ., with sufficient accuracy for most purposes. The air correction so calculated is given in the second column of the table.

Factors for calculating CO_2 content determined by volumetric apparatus with blood or plasma samples of 1 ml. (from Van Slyke and Stadie) are shown in this table:

Temperature $^\circ\text{C}$	Air in extracted gases from plasma and water. Subtract from observed air / CO_2 volume if CO_2 and air are measured together.	Factor by which ml. of CO_2 extracted from 1 ml. of plasma or blood is multiplied to give Volumes of CO_2
22	0.045	$96.0 \times B^*/760$
23	0.045	$95.4 \times "$
24	0.045	$94.8 \times "$
25	0.044	$94.2 \times "$
26	0.044	$93.6 \times "$
27	0.044	$93.1 \times "$
28	0.043	$92.4 \times "$
29	0.043	$91.8 \times "$
30	0.043	$91.2 \times "$
31	0.043	$90.6 \times "$
32	0.042	$90.0 \times "$
33	0.042	$89.4 \times "$
34	0.042	$88.8 \times "$

*B = observed barometric pressure

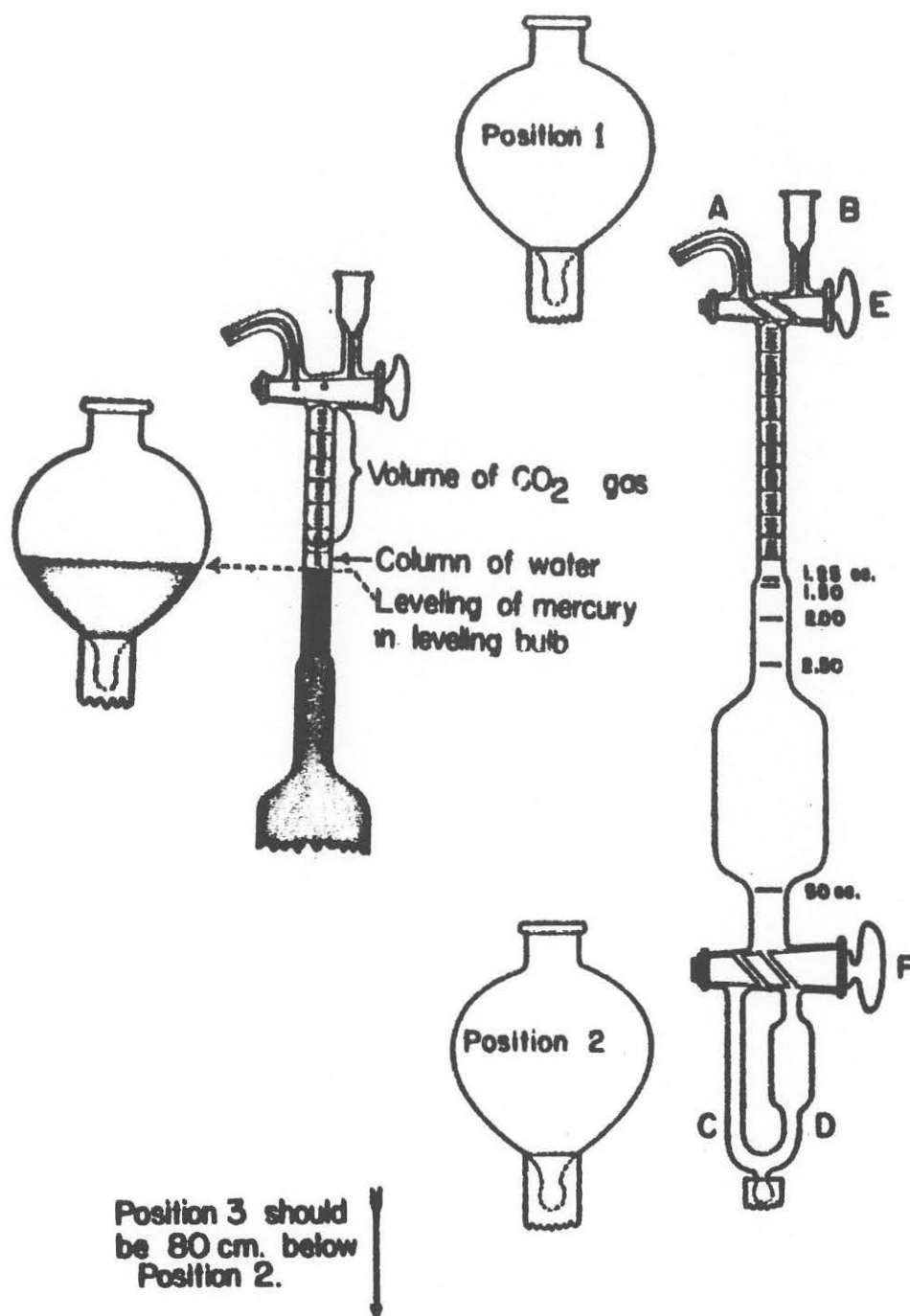


Figure 23. Van Slyke-Cullen Volumetric CO_2 Apparatus.

Chart for Rapid Calculation of Carbon Dioxide Values

B. P. = 753-767 mm. Hg.; Temperature = 23-25°C.

Volume of gas as read	Vol. %	meq CO ₂ per liter	Volume of gas as read	Vol. %	meq CO ₂ per liter
1.00	90.3	39.3	.60	52.3	22.7
.99	89.3	38.8	.59	51.3	22.3
.98	88.4	38.4	.58	50.4	21.9
.97	87.4	38.0	.57	49.4	21.5
.96	86.5	37.6	.56	48.5	21.1
.95	85.5	37.2	.55	47.5	20.7
.94	84.6	36.8	.54	46.6	20.3
.93	83.6	36.3	.53	45.6	19.8
.92	82.7	36.0	.52	44.7	19.4
.91	81.7	35.5	.51	43.7	19.0
.90	80.8	35.1	.50	42.8	18.6
.89	79.8	34.7	.49	41.8	18.2
.88	78.9	34.3	.48	40.9	17.8
.87	77.9	33.9	.47	39.9	17.3
.86	77.0	33.5	.46	39.0	17.0
.85	76.0	33.0	.45	38.0	16.5
.84	75.1	32.7	.44	37.1	16.1
.83	74.1	32.2	.43	36.1	15.7
.82	73.2	31.8	.42	35.2	15.3
.81	72.2	31.4	.41	34.2	14.9
.80	71.3	31.0	.40	33.3	14.4
.79	70.3	30.6	.39	32.2	14.0
.78	69.4	30.2	.38	31.4	13.7
.77	68.4	29.7	.37	30.6	13.3
.76	67.5	29.3	.36	29.5	12.8
.75	66.5	28.9	.35	28.5	12.4
.74	65.6	28.5	.34	27.6	12.0
.73	64.6	28.1	.33	26.6	11.7
.72	63.7	27.7	.32	25.7	11.2
.71	62.7	27.3	.31	24.7	10.7
.70	61.8	26.9	.30	23.8	10.3
.69	60.8	26.4	.29	22.8	9.9
.68	59.9	26.0	.28	21.9	9.5
.67	58.9	25.6	.27	20.9	9.1
.66	58.0	25.2	.26	20.0	8.7
.65	57.0	24.8	.25	19.0	8.3
.64	56.1	24.4	.24	18.1	7.9
.63	55.1	24.0	.23	17.1	7.4
.62	54.2	23.6	.22	16.2	7.0
.61	53.2	23.1	.21	15.2	6.6

Volume of gas as read meq CO₂ per liter

.20	14.2	6.2
.19	13.3	5.8
.18	12.4	5.4
.17	11.4	5.0
.16	10.5	4.6
.15	9.5	4.1
.14	8.6	3.7
.13	7.6	3.3
.12	6.7	2.9

Assumptions:

1. Barometric pressure varies between 753-767 mm. Hg.
2. Temperature varies between 23° and 25° C.

Note:

If plasma is allowed to stand exposed to the air while in contact with its erythrocytes, even more drastic changes will occur due to the Hamburger effect (the chloride shift). (Such changes are irreversible if the red blood cells are then removed.) This may best be explained by the following logical (not chronological) series of events.

In tissues:

1. CO₂ having been formed by the metabolic activity of the cells, passes through the intra-cellular fluid and then into the plasma. From the plasma the CO₂ passes into the red blood cell.

2. In the red blood cell, it forms H₂CO₃ by reacting with H₂O, catalyzed by carbonic anhydrase. $\text{H}_2\text{O} + \text{CO}_2 \longrightarrow \text{H}_2\text{CO}_3$

3. The newly formed H₂CO₃ produces little or no change in the erythrocyte pH because of the availability of potassium ion (K⁺) liberated by the conversion (by loss of O₂) of the stronger acid HHbO₂ (oxyhemoglobin) to the weaker acid HHb (reduced hemoglobin).

4. The newly formed KHCO₃ ionizes to form K⁺ and HCO₃⁻. Most of the HCO₃⁻ diffuses back into the plasma.

5. Since K⁺ as a cation is unable to diffuse with HCO₃⁻, an equivalent quantity of Cl⁻ diffuses from the plasma into the erythrocyte to maintain electrical neutrality.

In the lungs:

The reverse of the above events takes place.

1. HHb accepts oxygen and becomes a stronger acid, HHbO₂ which recaptures K⁺ and reforms H₂CO₃ which breaks down to form CO₂ and water.

2. The CO₂ diffuses from the cell into the plasma and out into the alveolar spaces.

3. Then more HCO₃⁻ diffuses back into the cell and Cl⁻ diffuses out.

4. Thus the overall effect is the loss of CO₂ to the lungs from the plasma HCO₃⁻ via the red cells (where HCO₃⁻ becomes CO₂).

When CO_2 in plasma is determined without the introduction of alkali, it is not necessary to wash the apparatus out between analyses. The slight amount of CO_2 remaining dissolved in the form of acidified solution which adheres to the chamber walls is negligible. After the measurement the contents are expelled into waste jar by elevating the leveling bulb and turning stopcock as to connect with waste jar. Air diffuses through rubber tubing slowly and may gradually accumulate in the apparatus. A blank extraction of 2.5 ml. of the lactic acid should yield no more extracted gas than the volume indicated in the second column of the above table.

12. The best procedure is to absorb the CO_2 by alkali. This absorption must be done when whole blood is analyzed. After the measurement of total gas volume, V_1 , the leveling bulb is lowered so that a partial vacuum is obtained, the gas space being increased to about 5 ml. in the chamber. The lower stopcock is now closed and 2 ml. of 1 N NaOH solution (see "Comments" below) is placed in the cup of the apparatus. One ml. of alkali is allowed to flow slowly into the chamber, at least 30 seconds being taken for its admission. After the alkali is admitted, the upper cock is sealed with mercury. The solution is now allowed to drain for one minute. (Do not shake.)

13. The gas volume is now brought to atmospheric pressure in the same manner as before and its volume is read. Call this volume V_2 . The difference, $V_1 - V_2$, is the ml. of carbon dioxide obtained from 1 ml. of serum at $t^\circ\text{C}$. and B mm. Hg. pressure. This value (ml. of CO_2) is multiplied directly by the factor indicated in the third column of the above table.

Calculation:

Example:	If $V_1 = 0.72$ ml.	Barometer	750 mm. Hg
	$V_2 = 0.05$ ml.	Factor	= $94.2 \times 750/760$
	$V_1 - V_2 = 0.67$ ml.	$t^\circ\text{C}$	= 25°C .

$$\begin{aligned}\text{Volumes \% CO}_2 &= 0.67 \times 94.2 \times 750/760 \\ &= 62.3 \text{ Volumes \%}\end{aligned}$$

Preparation of apparatus:

If this is kept clean, with well greased stopcocks, the only preparation necessary is to test for leaks. This test should be routine, and should never be omitted. It is performed by sealing both capillaries of the top stopcock with mercury, closing it, and drawing a vacuum to the 50 ml. mark. The stopcock grease is not strong enough to prevent leakage unless the capillaries and the bores in the plug are filled with mercury. Therefore, it is important that the stopcock be sealed with mercury before it is put under reduced pressure. After being drawn to the 50 ml. mark, the mercury is then allowed to rise to the top cock and to strike it gently. A sharp click should be produced. A muffled or soft click is an indication of the presence of air.

Precautions to be observed in handling blood gas apparatus:

1. When the apparatus is not in use the reaction chamber should be filled with water.
2. The stopcocks should be well greased (avoid excess) so that the flow through the

system can be controlled smoothly. Do not allow the stopcocks to be in contact with alkaline solutions longer than is necessary. The instructor will demonstrate the proper method of greasing the stopcocks. The stopcock plug must always be handled a short distance above a desk (preferably wooden) so that there is no chance of its slipping from greasy fingers onto the floor. If the plug falls a few inches onto the desk it will not break.

3. Mercury is heavy. When you handle the leveling bulb make sure that you have a good hold on it. When you set the leveling bulb down in the iron ring holders do it with care and make sure that it is secure before you let go.

4. Mercury is expensive. Please take care not to waste it and particularly avoid spilling of mercury into the sinks. If any mercury is spilled on desk, floor or sink, recover it and place it in the waste jar provided with each apparatus. This mercury is not thrown out. It is carefully cleaned and used again.

5. Mercury forms amalgams with the noble metals. Therefore it is suggested that all jewelry, including rings, wrist watches, and gold-trimmed fountain pens be removed from your person while working with this apparatus.

6. Please leave the apparatus at the end of the day filled with water as you found it at the beginning of the period.

Interpretation:

See Appendix for discussion of electrolyte balance.

Note: For rapid calculation of CO_2 in volumes % and meq./liter when the measurements are made at close to 760 mm. Hg. and 24°C . see table p. 133.

CARBON DIOXIDE

B. MANOMETRIC PROCEDURE

Principle:

Carbon dioxide is liberated from plasma by lactic acid (along with small amounts of other gases, such as O_2 and N_2). The pressure at a constant volume (2 ml.) is measured before and after absorption of the CO_2 with NaOH. The difference in pressure may be converted by an appropriate factor to volumes per cent CO_2 or to meq. per liter CO_2 .

Reagents:

As under the volumetric method.

NaOH 1 N (deaerated)

Prepare as follows:

1. Allow 25 ml. of solution to run into the chamber. Seal the stopcocks with mercury and evacuate to the 50 ml. mark.
2. Shake for 3 minutes and allow the air to escape through the cup by raising the mercury reservoir.
3. Reseal and repeat at least twice more until no measureable amount of residual air remains.
4. Transfer the deaerated NaOH into a storage chamber with stopcock under oil and seal the tip of the storage chamber by immersing in mercury.

Procedure:

1. The extraction chamber is cleaned by the use of 10-15 ml. of water and 1 ml. of 1 N lactic acid shaken under vacuum, and the cleaning fluid is ejected through the cup.
2. A drop of caprylic alcohol is drawn into the capillary above the top stopcock avoiding the entrance of air.
3. Deaeration of the lactic acid: 7.5 ml. of 0.1 N lactic acid is placed in the cup and drawn into the extraction chamber. The capillary of the cup and the stopcock are then sealed with mercury and the mercury meniscus is lowered to the 50 ml. mark. Shake for 3 minutes. The lower stopcock is now opened and the mercury is allowed to slowly rise. The mercury reservoir is now raised to the top position and the bottom stopcock closed.
4. Introduction of plasma sample: The top stopcock is opened to the cup and the bottom stopcock opened slowly to allow the lactic acid solution to rise to the 6 ml. mark on the cup (5 ml. for the blank "c" correction). The bottom stopcock is then closed and the mercury reservoir is lowered to the rest position. The sample is then delivered using a 1 ml. pipet with or without a rubber tip. The level of fluid in the cup is then lowered to exactly 5 ml. by the use of the bottom stopcock and the capillaries are then sealed with mercury, allowing a few drops of mercury to enter the extraction chamber.
5. Extraction of the gas: With the top stopcock closed and sealed with mercury,

the mercury meniscus is lowered to the 50 ml. mark. The bottom stopcock is closed and the extraction chamber is shaken for 3 minutes.

6. Reading of the pressure P_1 : P_1 is read by allowing the solution to rise slowly and evenly to the 2 ml. mark. If any bubbles are present they must be allowed to break before the reading is made. At the same time the temperature is recorded. To insure complete gas extraction the mercury meniscus is again lowered to 50 ml. and the extraction chamber is shaken for 3 minutes, and the reading P_1 is repeated.

7. Reading of the pressure P_2 : The solution is slowly lowered to the middle of the large bulb of the extraction chamber and approximately 2 ml. of 1 N NaOH (deaerated) is introduced into the cup. The top stopcock is carefully opened, care being taken to avoid the introduction of an air bubble. 1 ml. of the deaerated 1 N NaOH is allowed to run slowly (30-60 seconds) into the chamber. The capillaries are again sealed with mercury allowing a few drops to enter the chamber. Allow to stand for 1 minutes for full drainage, then allow the solution to rise slowly and carefully to the 2 ml. mark. Read the manometer P_2 .

8. Determination of the "c" correction factor: Proceed as under procedure 1-3 but allow the deaerated lactic acid to rise to the 5 ml. mark in the cup, thus leaving 2.5 ml. of lactic acid in the extraction chamber. The capillaries are sealed with mercury, and a P_1^C reading taken with the solution meniscus at 2 ml. 1 ml. of deaerated 1 N NaOH is added as under Step 7 and a P_2^C reading taken. The difference $P_2^C - P_1^C =$ "c", the correction factor.

Calculation:

$$P_{CO_2} = P_1 - P_2 - c$$

For the above given solution volumes and for various temperatures, the factors are given in the table.

Temperature °C	CO ₂		Temp. °C	Factor	
	Vol. %	meq./l		Vol. %	meq./l
15	27.35	12.44	26	25.81	11.75
16	27.19	12.38	27	25.69	11.69
17	27.04	12.30	28	25.57	11.64
18	26.90	12.23	29	25.45	11.60
19	26.75	12.15	30	25.33	11.56
20	26.62	12.10	31	25.22	11.50
21	26.48	12.04	32	25.11	11.44
22	26.34	11.98	33	25.00	11.38
23	26.20	11.92	34	24.89	11.32
24	26.07	11.86			
25	25.94	11.80			

$$P_{CO_2} \times (\text{factor}_1/100) = \text{volumes per cent CO}_2$$

$$P_{CO_2} \times (\text{factor}_2/100) = \text{meq. CO}_2/\text{liter}$$

Example:

$$P_1 = 348.7 \text{ mm.}$$

$$P_2 = 146.4 \text{ mm.}$$

$$c = 0.5 \text{ mm.}$$

$$\text{Temperature} = 24^\circ\text{C.}$$

$$P_{\text{CO}_2} = 348.7 - 146.4 - 0.5$$

$$P_{\text{CO}_2} = 201.8 \text{ mm.}$$

$$\text{Factor}_1 = 26.07; \text{Factor}_2 = 11.86$$

$$201.8 \times (26.07/100) = 52.6 \text{ vol. \% CO}_2$$

$$201.8 \times (11.86/100) = 23.9 \text{ meq. CO}_2/\text{liter}$$

Interpretation: See Acid Base Balance, p. 321.

Alternate Procedure (1):

If 0.2 ml. of 5 N NaOH is used to absorb the CO_2 in the air phase, it is not necessary to deaerate the alkali since it dissolves only about 10 per cent as much air as the 1 N NaOH.

Alternate Procedure (2):

1.5 ml. of lactic acid and 1.0 ml. of plasma may be extracted as outlined in the volumetric procedure and P_2 measured with the gas volume at 2.0 ml. The gas is then ejected without loss of fluid, the capillary is resealed with mercury and the pressure measured again at 2.0 ml. gas volume. At $20-28^\circ\text{C}$, the following equation applies:

$$(P_1 - P_2) \times 0.256 - 8.6 = \text{Vol. \% CO}_2$$

CARBON MONOXIDE IN BLOOD

Micro Gasometric Estimation

References:

1. Roughton, F. J. W., and Scholander, P. F., "Micro Gasometric Estimation of the Blood Gases," J. Biol. Chem., **148**, 551-63 (1943).
2. Kaye, Sidney, Handbook of Emergency Toxicology, Charles C. Thomas - Publisher, Springfield, Illinois, pp. 134-40 (1954)

Principle:

In the microgasometric technique for estimating the carbon monoxide in a drop of blood, 40 cmm. of blood are mixed in a 1 ml. syringe with ferricyanide containing potassium bicarbonate and saponin. An acetate buffer is then added. The CO_2 which is evolved upon shaking provides a gas phase for the extraction of the other gases of the blood and reagents; i. e., O_2 , CO , and N_2 . After the extraction is complete, the CO_2 and the extracted O_2 are absorbed by alkaline pyrogallol; the small bubble which remains is measured in a graduated capillary attached to the nozzle of the syringe. The CO is absorbed by Winkler's solution and the gas bubble measured again. From the difference between the two readings the CO content of the blood may be calculated. No blank correction is required for the reagents since the blood itself is the only source of CO .

Apparatus:

A special syringe analyzer and pipets are required. These are described in the original reference. They may be obtained by writing James D. Graham, 11 Montwell Avenue, Haddonfield, New Jersey.

Syringe, luer, 2 ml. and 10 ml.

Needle, hypodermic, 21-gage.

Reagents:

1. Distilled water.
2. Caprylic alcohol.
3. Ferricyanide solution. 2.5 grams of $\text{K}_3\text{Fe}(\text{CN})_6$, 0.6 grams of KHCO_3 , and 0.1 grams of saponin are ground in a mortar and dissolved in water to make up to 10 ml. This solution should be renewed every three days.
4. Acetate Buffer. 70 grams of sodium acetate ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) are dissolved in 100 grams of water and 15 mls. of glacial acetic acid then added.
5. Urea. Dissolve 45 grams of urea in water and make up to 100 mls.
6. Winkler's Solution. 20 grams of cuprous chloride (CuCl), 25 grams of ammonium chloride, and 75 grams of water are placed in a bottle just large enough to contain them. The bottle is corked, shaken with as little air as possible, and the precipitate allowed to settle. A coil of copper wire is placed in the solution which is then covered with a layer of paraffin oil. After some time the reagent becomes colorless.
7. Pyrogallol Solution. 15.0 grams of powdered pyrogallol ($\text{C}_6\text{H}_3(\text{OH})_3$) are added to 100 mls. of 20 per cent NaOH in a rubber stoppered bottle, and covered with a layer of paraffin oil. The pyrogallol is dissolved under the oil by stirring with a glass rod.

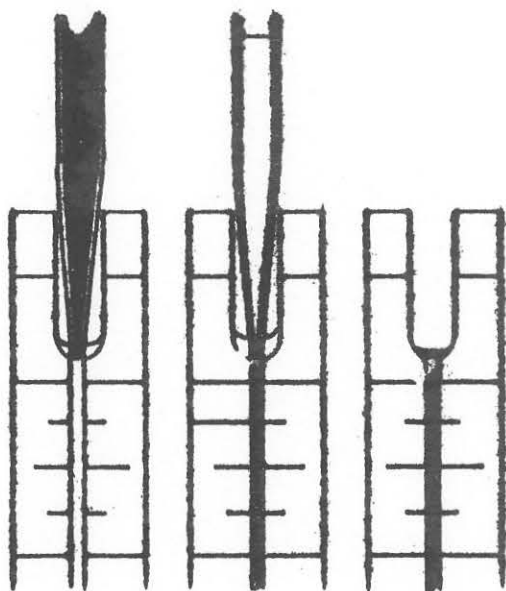


Figure a. Transfer of blood from pipet to capillary.

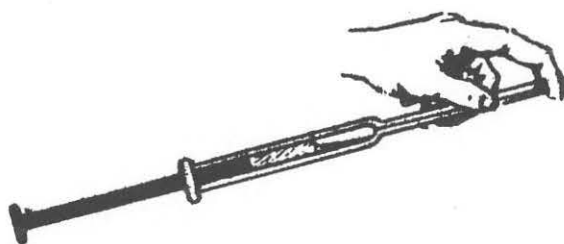


Figure b. Shaking of syringe and extraction of gases.

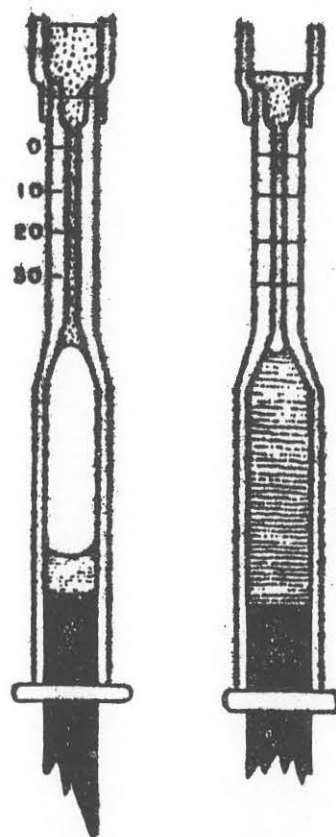


Figure c. Absorption of CO_2 by alkaline reagent.

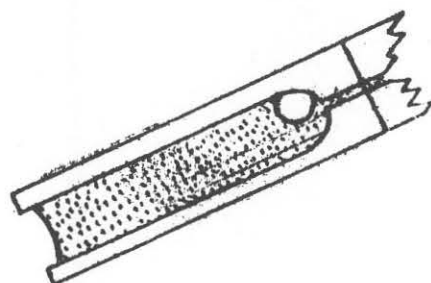


Figure d. Absorption of CO_2 by Winkler's solution.

These illustrations have been adapted from Reference 1.

Figure 24. Carbon monoxide analysis.

Figure 34. Carbonaceous residue.

These illustrations have been adapted from Helander 1.

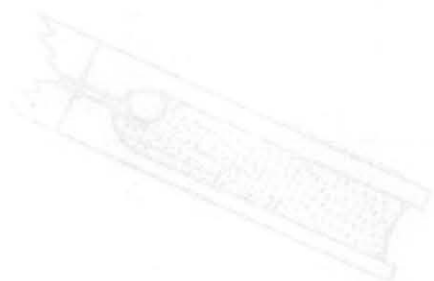
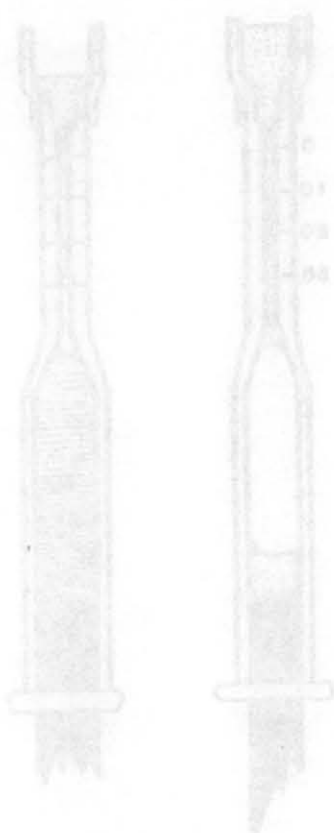
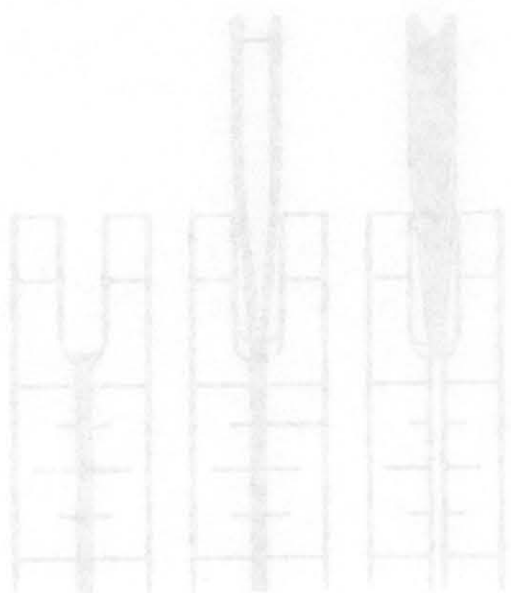
Figure 3. Absorption of CO_2 by Weiser's solution.Figure 5. Absorption of CO_2 by alkaline reagent.

Figure 6. Sealing of syringe and activation of tissue.



Figure 2. Transfer of blood from syringe to capillary.



Blood Samples:

A blood specimen of approximately 5 ml. is collected by sterile technique, with a minimum of exposure to the air during all steps of the collection. The specimen should be drawn and transferred to the container slowly to prevent aeration. It is then placed in a clean screw-cap test tube containing 5 mg. of lithium oxalate. Overlay the specimen with 1 ml. of liquid petrolatum and gently stir with a clean rod to effect solution of the oxalate. Fill the test tube with liquid petrolatum and screw on the cap.

Procedure:

1. The syringe is held vertically and any liquid in the cup of the syringe is removed by vacuum suction. The ferricyanide solution is drawn to the bottom of the syringe and expelled through the cup and removed. This procedure is repeated twice with fresh lots of ferricyanide, the dead space finally being left full of ferricyanide without trapping any air bubbles. No grease or oil is used in the syringe.
2. The glass cup is filled to the mark with ferricyanide and the latter is drawn down to the bottom of the cup.
3. A drop of caprylic alcohol is deposited on the bottom of the cup.
4. The pipet is filled to the mark with blood, wiped, and held at a slight angle to the horizontal so that the blood does not run out when both ends of the pipet are open to the air. With the syringe at the same angle, the pipet is cautiously introduced into the glass cup, and its tip pressed snugly, but not too vigorously, against the bottom of the cup. (Fig. 24a, p. 140a).
5. By pulling out the plunger gradually the blood is slowly and evenly drawn into the capillary, followed by a bubble of air about 1 mm. in length. If the tip is properly ground, and the right amount of pressure is applied, no appreciable caprylic alcohol is drawn in during this step. The bubble of air prevents any blood being sucked back into the tip when the pipet is removed.
6. The pipet is quickly removed and the bubble of air is then expelled through the caprylic alcohol, with the aid, if necessary, of a piece of fine wire or by tapping the capillary.
7. A trace of caprylic alcohol, i.e., about two division lengths of the capillary, is drawn onto the top of the blood and the rest of the caprylic alcohol is removed from the cup by suction.
8. The cup is filled to the mark with acetate buffer and the latter drawn down to the bottom of the cup.
9. The cup is then immediately filled to the top with 45 per cent urea, and then closed firmly with the finger.
10. The closed apparatus is vigorously shaken in the horizontal position, the plunger being gradually drawn out as the CO_2 and the other gases are evolved, the gas pressure being kept roughly at atmospheric. (Fig. 24b.) The total volume evolved should be about 0.75 cc. If the amount is appreciably more or less than this the concentration of KHCO_3 in the ferricyanide reagent should be correspondingly adjusted. Shaking is continued for a total of 2 minutes.
11. The finger is cautiously released, the syringe plunger being manipulated so as to keep the gas meniscus in the capillary. A small amount of urea is run down into

the capillary and left there until the walls are perfectly clean.

12. Three-quarters of the urea in the glass cup is removed, and the rubber cup adjusted and filled with pyrogallol solution without trapping air bubbles.

13. A little pyrogallol is drawn into the syringe. This absorbs some CO_2 and O_2 , causing a partial vacuum which quickly sucks in more pyrogallol until only a small bubble consisting of N_2 and CO (if any was originally present in the blood) is left at the top of the syringe. (Fig. 24c.) The absorption takes a few seconds and just before it is complete the residual bubble is screwed slowly and carefully up into the capillary by manipulation of the plunger.

14. The rubber cup is removed, and the glass cup is emptied by suction.

15. The capillary is placed for half a minute in a beaker of water at room temperature.

16. It is then removed, dried by light wiping, care being taken that the capillary is not handled, and the volume of the bubble read, V_1 divisions.

17. The glass cup is flushed clean with water and left filled. About three-quarters of this water is pulled quickly down into the syringe, forming a layer on top of the heavier blood mixture. The bubble, with clean water below it, is then at once run up to the top of the capillary.

18. The glass cup is emptied of water and filled with Winkler's solution.

19. The syringe is then pointed with the cup downwards, the capillary making a slight angle with the horizontal. By cautiously screwing in the plunger the gas bubble is driven out into the glass cup where it rests near the junction of the capillary and the cup. (Fig. 24d.) As soon as the bubble is free in the cup, Winkler's solution is sucked behind it so as to half fill the capillary. Gentle rotation for a few seconds completes the absorption of the CO . The syringe is then turned to the vertical position with the cup downwards and the gas bubble is sucked back into the capillary and its volume, V_2 , measured as above.

20. To wash the instrument, the plunger is pulled out under a stream of running water, and the blood mixture poured out. The syringe is filled and emptied several times with water before the plunger is restored. The plunger should never be forced. If there is resistance due to precipitates inside it the whole syringe should be re-cleaned. Occasional cleaning with chromic acid is recommended.

Calculations:

The carbon monoxide content of the blood in volume per cent equals $(V_1 - V_2) \times f$, where f is the correction factor for temperature, aqueous vapor pressure, and barometric pressure. In COHb estimations at saturations below 8 volumes per cent, at room temperature and at sea level it is often in practice accurate enough to let $f = 0.9$.

Example:

Reading of V_1 -- -5.2

Reading of V_2 -- -4.7

$$0.9(5.2 - 4.7) = \text{volumes per cent carbon monoxide}$$

$$0.9(0.7) = 0.63 = \text{volumes per cent carbon monoxide}$$

At the U. S. Naval Medical School, further calculations are made as follows:

The hemoglobin of the blood is determined by any convenient method, and this value is multiplied by 1.36 (conversion factor - one gram hemoglobin equals 1.36 volumes per cent carbon monoxide) to obtain the carbon monoxide capacity of the blood. The carbon monoxide content multiplied by one hundred, then divided by the carbon monoxide capacity gives the percentage of saturation of the blood specimen.

Example:

Hemoglobin - 14 grams

Carbon monoxide content - - 0.63 volume per cent

Carbon monoxide capacity - - $14 \times 1.36 = 19.0$

Carbon monoxide saturation = $\frac{0.63 \times 100}{19} = 3.3$ per cent saturation

Notes:

1. One objection to this method is that the alkaline pyrogallol solutions used may evolve traces of CO. This tendency diminishes with the strength of alkali and pyrogallol used, but increases with the partial pressure of O₂ and the time of contact of the pyrogallol solution with the gas phase. The CO is thus apparently a secondary product of the reaction between O₂ and pyrogallol. With the amounts of O₂ yielded by 40 c. mm. of blood this pyrogallol solution has not been found to evolve any CO when used in the syringe technique.

2. The alkaline pyrogallol solution must be removed into the syringe from under oil without exposure to air.

3. A drop of caprylic alcohol is introduced between the barrel and plunger of the syringes used for the reagents, to lessen the exposure to air, and also to prevent freezing of syringe due to the drying of some of the reagents.

Interpretation:

The normal carbon monoxide blood level is 0 to 2 per cent for non-smokers and 1 to 3 for smokers. The minimum lethal dose is a 40 per cent carboxy-hemoglobin saturation. If death is due to carbon monoxide, samples drawn from the body weeks later will still so indicate since putrefaction will not affect the carbon monoxide level.

For a blood sample of 40 c. mm as described in the technique the accuracy of a single determination is 0.15 to 0.20 volume per cent.

CEPHALIN FLOCCULATION Serum

Reference:

Hanger, F. M., Jour. Clin. Invest., 18, 261 (1939). Am. J. Med. Sci. 202, 48 (1941).

Principle:

Disturbances of the liver parenchymal cells may be detected by noting the capacity of blood serum to flocculate a colloidal suspension of cephalin and cholesterol.

Reagents:

1. Cephalin-cholesterol mixture. This consists of 100 mg. of partially oxidized cephalin and 300 mg. of cholesterol. The mixture is purchased from suppliers such as Difco Laboratories. Prepare the ether mixture by adding 5 ml. of anesthetic ether per unit to effect solution of the contents. If turbidity persists, add one drop of distilled water to obtain a clear solution. This solution constitutes the stock ether antigen of Hanger and is stable for months if kept tightly stoppered to prevent evaporation. Keep refrigerated.

2. Preparation of test antigen from the cephalin-cholesterol mixture. Add slowly with stirring, 0.5 ml. of stock ether-antigen to 18 ml. of distilled water at 65-70° C. Then heat slowly to boiling. Simmer until the volume is reduced to 15 ml. This should result in a stable, milky emulsion. Prepare fresh each day.

Procedure:

Place 1 ml. emulsion in a test tube containing 0.2 ml. of the patient's serum (not over 24 hours old) and 4 ml. 0.85% saline. Mix well by shaking.

Control tube - 4 ml. saline + 1 ml. emulsion. If possible run positive and negative control sera. Read after 24 and 48 hours. Record results.

Interpretation:

24 hours = negative

48 hours = negative to 2 +

A negative result is of value in excluding parenchymal hepatic disease. Some workers believe that a positive result is due to various combinations of three conditions:

1. Increase of gamma globulin
2. Decrease of serum albumin
3. Change in properties of serum albumin.

negative = milky solution - no visible flocculation

1 + = milky solution - faint flocculation - dispersed

2 + = milky solution - definite flocculation - dispersed

3 + = milky solution - partially settled flocculation

4 + = clear solution - complete flocculation

CHLORIDES
Serum, Spinal Fluid, and Urine

References:

1. Shales, O., and Shales, S.S., J. Biol. Chem., 140, 879 (1941)
2. Franco, V., and Klein, B., J. Lab. Clin. Med., 37, 950 (1951)
3. Saifer, A. and Hughes, J., J. Biol. Chem. 129, 273 (1939)
4. Whitehorn, J.C., J. Biol. Chem., 45, 449 (1921)
5. Wilson, D.W., and Ball, E.G., J. Biol. Chem., 79, 221 (1928)
6. King, E.J., and Bain, D.S., Biochem. J., 48, 51 (1951)

Method I. (Reference 1)

Principle:

This is a mercurimetric procedure. As with silver ions, chloride also combines with mercuric ions according to the following equation:



No precipitate is formed, contrary to the effect of silver ions forming insoluble silver chloride but the mercuric chloride is only very slightly dissociated into ions, and the end point is recognized by the appearance of Hg^{++} ions in solution. The recognition of Hg^{++} is made possible by various indicators such as urea, sodium nitroprusside, etc., but in this method use is made of s-diphenylcarbazone (EK Co. #4459) which changes from colorless or light yellow to an intense blue when combined with Hg^{++} . It is essential that the pH at the end point be between 1 and 2.

Apparatus:

1. Flasks 25 ml. Erlenmeyer.
2. Microburet calibrated in 0.01-0.005 ml. 100 drops should equal 1 ml.

Reagents:

1. Mercuric nitrate solution 1.6-1.8 g. $\text{Hg}(\text{NO}_3)_2$ are dissolved in a few hundred ml. of water with the addition of 20 ml. of 2 N HNO_3 . Make up to 1000 ml. with water.
2. Standard sodium chloride solution.

Sodium chloride A.R. is dried at 120°C . and 584.5 mg. are dissolved in water and made up to 1000 ml. This solution contains 0.01 milliequivalents of chloride/ml. It is used for the standardization of the mercuric nitrate solution each day.

3. Indicator--100 mg. of diphenylcarbazone (EK Co. #4459) is dissolved in 100 ml. of 95% alcohol and stored in the dark, preferably refrigerated. If the end point becomes sluggish, discard the solution; it is better to prepare new solution monthly. (It has been suggested that a sharper endpoint is achieved by using diphenylcarbazide-sulfonic acid.)

The stability of the diphenylcarbazone solution is improved by the use of a product obtained by recrystallization as follows:

Saturate 200 ml. of 95% ethyl alcohol with the commercial s-diphenylcarbazone. Filter or decant the solution. Add water to the solution gradually until the maximum turbidity is obtained. Allow to stand at refrigerator temperature overnight. Collect the crystals by filtration using a Buchner funnel and a hard (Whatman #50) filter paper. Dry in a desiccator, do not use heat. Store the solid in the refrigerator.

Procedure:

1. Whole serum: To 2.0 ml. of water in a 25 ml. Erlenmeyer flask is added 0.20 ml. of serum by 8 times rinsing from a 0.200 ml. "to contain" pipet, and 0.06 ml. of indicator (4 drops). The color in this slightly turbid mixture is salmon red, which changes to a deep violet, and then to a light yellow, as titration with the acid mercuric nitrate solution proceeds and at the end-point a change to a pale violet occurs, which can be seen quite easily. The drops from the buret should be very small, about 100 to the ml.

2. Serum filtrates: To 2.0 ml. of filtrate (= 0.2 ml. serum) in a 25 ml. Erlenmeyer flask is added 4 drops of indicator solution and the titration carried out as noted above. The color changes are somewhat different but at the end point changes to a bright blue.

Calculation:

T_s = ml. mercuric nitrate solution required for titration of 2.0 ml. of standard sodium chloride solution (= 0.02 meq.). T_u = ml. mercuric nitrate reagent required to titrate 0.2 ml. of serum (or equivalent filtrate).

$$C_s = \text{meq. Cl}^- \text{ in 2.0 ml. of standard NaCl} = 0.02$$

$$(T_u/T_s) \times C_s = \text{meq. Cl}^- \text{ in 0.2 ml. serum}$$

$$(T_u/T_s) \times C_s \times \frac{1000}{0.2} = \text{meq. Cl}^-/\text{liter serum} = (T_u/T_s) \times 100$$

$$\text{or } (T_u/T_s) C_s \times \frac{100}{0.2} \times 35.5 = \text{mg. \% (as chloride ion)} = (T_u/T_s) \times 355$$

$$(T_u/T_s) \times 585 = \text{mg. \% as NaCl}$$

Note: In order to increase the volumes measured in titration, we actually use 5 ml. of a 1:10 filtrate of serum (equivalent to 0.5 ml. of serum) and 5 ml. of the standard containing 0.05 meq. Cl^- .

For urine and spinal fluid using a 0.5 ml. samples and the T_s of 2 ml. of standard.

$$(T_u/T_s) \times 4 = \text{meq. Cl}^-/100 \text{ ml. sample}$$

$$(T_u/T_s) \times 40 = \text{meq. Cl}^-/\text{liter}$$

$$(T_u/T_s) \times 142 = \text{mg. Cl}^-/100 \text{ ml.}$$

$$(T_u/T_s) \times 234 = \text{mg. NaCl}/100 \text{ ml.}$$

Notes:

The authors and others state that direct titration of serum gives results 1-3 meq./liter higher than the filtrates and ascribe this effect to the adsorption of Cl^- by the precipitate. However, the protein present in serum does affect the titration and filtrates should be used, for the greatest precision.

1. Hypodermic needles as buret tips are not satisfactory since the mercuric ion reacts with the metal in the tip.

2. In preparing the mercuric nitrate solution the amount of nitric acid specified should be used; otherwise the end point will not be sharp.

3. Urine and Spinal Fluid: To 0.5 ml. urine or spinal fluid add 5.0 ml. 0.05 N HNO_3 and titrate as above. Calculation as above except taking into consideration the volume of urine or spinal fluid used.

Chloride Method II. (Reference 4)

Principle:

The chloride is precipitated by the addition of a measured amount of silver nitrate. The excess silver is then titrated with NH_4CNS . The indicator used is ferric ammonium sulfate, which with excess thiocyanate gives the red color of $\text{Fe}(\text{CNS})_3$.

Reagents:

1. Silver nitrate - 5.813 g. AgNO_3 diluted to 1 liter with H_2O (distilled). Preserve in brown bottle. 1 ml. = 2.0 mg. NaCl.
2. Ammonium thiocyanate - Dissolve 3 g. NH_4SCN in 1 liter distilled H_2O . Standardize against AgNO_3 , using the technique given under procedure and adjust so that 5 ml. = 5 ml. AgNO_3 .
3. Ferric ammonium sulfate - solid.
4. HNO_3 concentrated.

Procedure:

Serum:

1. To 10.00 ml. of a tungstic acid filtrate of serum, add with stirring 5.00 ml. of silver nitrate solution. Add 4 ml. concentrated HNO_3 , mix and allow to stand for five minutes in the dark.

2. Add a few grains (about 0.3 g.) of ferric ammonium sulfate and titrate to the first reddish-brown endpoint lasting ten to fifteen seconds.

Spinal Fluid:

1. To 1.00 ml. (or 0.5 ml.) spinal fluid, add 9 ml. H_2O and 5.00 ml. AgNO_3 solution, mixing during the addition. Add 4 ml. concentrated HNO_3 , mix, add a few grains of solid ferric ammonium sulfate.

2. Allow to stand in the dark 5 minutes.

3. Titrate with ammonium thiocyanate solution to the first lasting reddish brown color.

Urine:

1. Dilute urine 1:10 (1 ml. / 9 ml. H_2O).

2. To 10.0 ml. of diluted urine add 5.00 ml. AgNO_3 and proceed as above under spinal fluid chloride.

Calculations:

If t is the volume in ml. of NH_4SCN used in the back-titration:

Then $(5 - t) \times 2 = \text{mg. NaCl in the sample.}$

$(5-t) \times 2/58.5 = \text{meq. Cl}^- \text{ in the sample and } (5-t) \times 2 \times 35.5/58.5 =$
mg $\text{Cl}^-/\text{sample}$

$(5-t) \times 2/58.5 \times 1000/0.5 = \text{meq. Cl}^-/\text{liter (using a 0.5 ml. sample)}$

Chloride Method III. (References 2 and 3)

Principle:

Serum, spinal fluid, and urines are deproteinized by the addition of the reagents of Somogyi (1945) ZnSO_4 and Ba(OH)_2 (see deproteinizing methods p. 79) and the filtrate is titrated with silver nitrate using dichlorofluorescein as an adsorption indicator. The precipitate AgCl with the first excess of Ag^+ becomes an ion, (AgClAg^+) which strongly adsorbs the indicator dichlorofluorescein, (yellow in solution) which turns to red on the solid phase.

Reagents:

1. Ba(OH)_2 0.086 N. Prepare by dilution of 0.3 N Ba(OH)_2 described under Method IV of deproteinizing methods, (add 2 ml. to 5 ml. of water). The Ba(OH)_2 is diluted at the time of use and kept protected from the CO_2 of the air.
2. ZnSO_4 , 5% - described in deproteinizing methods.
3. Standard NaCl - 1.000 g. dried reagent NaCl is dissolved up to 1000 ml. with water; 1 ml. = 1.0 mg. NaCl .
4. Silver nitrate 0.1 N. Weigh out exactly 8.4945 g. reagent grade silver nitrate, transfer to a 500 ml. volumetric flask and dilute to 500 ml. Keep in a brown bottle in the dark. This may serve as the primary standard instead of the NaCl .
5. Silver Nitrate 0.02 N. Prepared by dilution of the 0.1 N solution and standardized against NaCl , using the technique described for blood filtrates under procedure.
6. Dichlorofluorescein. 0.05% in 70% ethyl alcohol. Weigh out (analytical balance) 50 mg. of one mg. of dichlorofluorescein (EKCo. #373) dissolved in a mixture of 74 ml. of 95% ethyl alcohol and sufficient water to give 100 ml. (graduate).

Procedure:

1. Serum:

Add 7.0 ml. dilute Ba(OH)_2 solution to 1.0 ml. of clear serum; then add 2.0 ml. of ZnSO_4 solution, stopper and mix well by shaking. Centrifuge to pack precipitate well. Transfer 2.0 ml. aliquots to 25 ml. Erlenmeyer flasks; add 1 drop of indicator and titrate with 0.02 N AgNO_3 solution until the first pink is seen through the entire solution.

2. Spinal Fluid:

0.5 ml. of clear spinal fluid is treated with 3.5 ml. of dilute Ba(OH)_2 solution and 1.0 ml. ZnSO_4 solution, 2.0 ml. aliquots are titrated as described above.

3. Urine:

To 0.2 ml. urine in a pyrex test tube are added 3 drops of 3% H_2O_2 (chloride free). Heat tube in a boiling water bath for two minutes. Cool. Pipet into this tube 3.5 ml. dilute Ba(OH)_2 ; 1.0 ml. ZnSO_4 and 2.0 ml. H_2O . Mix well. Centrifuge and remove 3.0 ml. aliquots.

Calculation:

$$T_{Ag} = \text{ml. Silver Nitrate}(0.02 \text{ N})$$

Serum and Spinal Fluid:

$$T_{Ag} \times 0.02 = \text{meq. Cl}^-/\text{sample:} \quad \text{meq} \times 35.5 = \text{mg. Cl}^-$$
$$\text{meq} \times 58.5 = \text{mg. NaCl}$$

$$T_{Ag} \times 0.02 \times 1000/0.2 = \text{meq Cl}^-/\text{liter of serum}$$

$$T_{Ag} \times 100 = \text{meq Cl}^-/\text{liter}$$

Urine:

$$T_{Ag} \times 0.02 \times 6.85/3.00 \times 1000/0.2 \text{ meq. Cl}^-/\text{liter of urine}$$

$$T_{Ag} \times 228.3 = \text{meq Cl}^-/\text{liter of urine}$$

Chlorides Method IV. (Reference 6)

Principle:

Silver iodate (insoluble) reacts with chloride in solution to form a precipitate of silver chloride and leaves iodate in solution. $\text{NaCl} + \text{AgIO}_3 \rightarrow \text{AgCl} + \text{NaIO}_3$. The iodate in solution which is stoichiometrically equivalent to the chloride in the sample used, is determined by adding potassium iodide in acid solution and titrating the liberated iodine with thiosulfate. For reagents, procedure, and calculations, see the original reference.

Interpretation:

See Electrolyte Balance in Appendix.

SWEAT ELECTROLYTES

No special preparation of the patient is necessary. Wash the mid-back with distilled water and dry. Do not touch this area with your fingers. With forceps place a three inch square gauze pad (Curity brand) in a stoppered Erlenmeyer flask and weigh on an analytical balance. Still using forceps, place the pad on the back and cover with a slightly larger piece of plastic sheeting. Tape the sheeting to the back with eight strips of one inch adhesive tape - two strips of tape to each side of the sheeting. Place the patient in a plastic suit which has an elastic neck and a zipper in the front. Cover patient with one or two blankets. Note the time. At intervals note the amount of sweat condensed in the bag and if it appears sufficient remove patient from bag. The average collection time is 60 minutes (range - 15 minutes to 90 minutes). Remove the plastic sheeting. Then replace the sweat soaked gauze pad (use forceps) into the same stoppered Erlenmeyer flask and reweigh. The difference between the two weights, the wet minus the dry, represents the amount of sweat collected.

Procedure:

Add 20 ml. of distilled water to the flask containing the sweat-soaked gauze pad. Allow the sweat and distilled water to become well mixed. Now place 5 ml. of this diluted sweat sample in an evaporating dish. Add 10 drops of diphenylcarbazone indicator. Titrate to end point with standardized mercuric nitrate solution in the usual manner.

Sweat Chloride

Calculation:

If s is the weight of sweat collected, then:

$$\frac{20 \text{ ml. } \cancel{f} \text{ s}}{s} = \text{dilution factor (F}_d\text{)}$$

$$\text{Sweat Cl}^- (\text{meq. / L}) = (T_u / T_s) \times C_s \times \frac{1000}{5 / F_d}$$

Example:

Flask stopper, pad and sweat	53.2239 g.
Flask stopper and dry pad	51.7320 g.
Sweat (s)	1.4919 g.

$$\frac{20 \cancel{f} s}{s} = \frac{21.4919}{1.4919} = F_d = 14.4$$

Using 5 ml. of standard chloride and 5 ml. of diluted sweat: (cont.)

$$T_s = 4.988 \text{ ml.}$$

$$T_u = 0.63 \text{ ml.}$$

$$C_s = 0.05 \text{ meq. Cl}^-$$

$$\begin{aligned} \text{Sweat Cl}^- &= (0.63/4.988) \times 0.05 \times \frac{1000}{5/14.4} = \text{meq./l.} \\ &= 0.1263 \times 0.05 \times 2880 \\ &= 18.2 \text{ meq./liter} \end{aligned}$$

References:

1. Darling, R.C.: Am. J. Med. Sc., 225, 67 (1953).
2. di Sant'Agnese, P.A.: Pediatrics, 12, 549 (1953).
3. Schwachman, H.: American Academy of Pediatrics, Oct. 5, 1954, Chicago.
4. Schwachman, H.: Advances in Pediatrics, Vol. VII, 249-323, 1955.

Mucoviscidosis accounts for almost all cases of pancreatic insufficiency in children. The incidence in the population of the United States is between 1 in 600 and 1 in 10,000 live births, with a probable average incidence of 1 in 2,500. It is a familial disease displaying the characteristics of a mendelian recessive gene. Both parents must be carriers of the trait. In an affected family, the disease may occur in approximately 25% of the offspring, and two-thirds of the non-affected children may be carriers. Most patients with this disease have manifestations before 6 or 8 months of age.

The measurement of the sodium and chloride concentration in body sweat is a well established diagnostic procedure in the study of patients suspected of having Mucoviscidosis (cystic fibrosis of the pancreas). The salt concentration runs two to four times as high as that seen in a control group. This change is independent of the pulmonary or pancreatic process, and has no association with renal, adrenal, or gastrointestinal tract dysfunction. The excessive salt loss readily explains the abnormally high incidence of heat prostration in children with mucoviscidosis.

A value of sodium above 80 meq./L of sweat or a value of chloride above 70 meq./L of sweat is characteristic of the disease. A small percentage of parents and apparently well siblings of affected children will show such an elevation. The absence of perceptible sweating in the neonatal period makes this test invalid during the first three to five weeks of life.

Potassium content of sweat in patients with mucoviscidosis is also increased but is not sufficiently abnormal to be of any diagnostic value.

CHOLESTEROL AND ESTERS Serum

References:

1. Bloor, W.R. and Knudson, A., J. Biol. Chem., **27**, 107 (1916)
2. Bloor, W.R. and Pelhan, K.F., Allen, D.M., J. Biol. Chem., **52**, 191 (1922)
3. Shoenheimer, R., and Sperry, W.M., J. Biol. Chem., **106**, 745 (1934)
4. Sperry, W.M. and Webb, M., J. Biol. Chem., **187**, 97 (1950)
5. Carr, J.J., and Drecker, I.J., Clin. Chem., **2**, 353 (1956)

Method I (Reference 1)

Principle:

Serum is added to a mixture of alcohol and ether, which extracts the lipids and precipitates the protein.

Total Cholesterol

An aliquot of the alcohol-ether extract is evaporated to dryness, the cholesterol in the residue is extracted with chloroform, and color is developed by the Liebermann-Burchard reaction. Equimolecular amounts of free and ester cholesterol give identical amounts of color if the determination is made at 25°C. and the color is read at 45 minutes.

Cholesterol Esters

Digitonin is added to the alcohol-ether extract of serum, precipitating free cholesterol as the digitonide. The digitonide is insoluble in petroleum ether and remains behind while the esters are extracted with this solvent. The petroleum ether is then evaporated to dryness. The cholesterol esters in this residue are extracted into chloroform and the color developed as above.

Reagents:

The solvents mentioned may be satisfactory for use without distillation. This may be determined by repeated experimental determination of the slope and shape of the standard density vs. concentration (D/C) curve, and should include a density vs. time curve also. If a solvent requires purification, it should be redistilled.

1. Alcohol-(ethyl) 95%.
2. Ether-(diethylether).
3. Alcohol-ether. Three parts of 95% alcohol plus 1 part of ether (by volume).
4. Chloroform - may be used without redistillation but should be stored over anhydrous sodium carbonate to keep it dry and neutral in reaction.
5. Acetic anhydride reagent. In a dry container, place 10 ml. of glacial acetic acid, and 20 ml. acetic anhydride. Cool well in an ice water bath and add 2 ml. of ice cold concentrated sulfuric acid. Mix well. Allow to warm to room temperature before use.
6. Cholesterol standard solutions: Dissolve 40.0 mgm. cholesterol up to 500 ml. with alcohol-ether mixture. Keep tightly stoppered in a glass stoppered bottle. Prepare new standard monthly. 10 ml. = 0.8 mg. of cholesterol.

7. Petroleum ether (see page 157).

8. Digitonin - 1% solution in 95% ethyl alcohol. Digitonin dissolves slowly and may require heating in a water bath.

Procedure:

Note: All apparatus must be dry.

Preparation of protein-free lipid filtrate: In a 50-ml. volumetric flask place about 25 ml. alcohol-ether mixture. Add slowly with shaking, 2.0 ml. serum. Heat mixture to boiling in a waterbath, (no flame). Cool to room temperature. Dilute to volume with alcohol-ether. Mix. Filter through Whatman #43 filter paper (or other fat-free filter paper), into a glass-stoppered bottle, avoiding evaporation of solvent.

Total Cholesterol:

1. Transfer 10.0 ml. of filtrate to a 100 ml. beaker. Evaporate just to dryness, using a gentle (l) air current if desired, dry under infra-red lamp 10 minutes.

2. Extract the residue, first with 3 ml. then with 2 ml. four times with chloroform (3, 2, 2, 2, 2,). Each time rinse down the sides, cover with a watch glass, boil until a drop of condensed CHCl_3 drops from the watch glass, using a hot plate set at gentle heat (or a waterbath).

3. Filter each extract through a funnel with a bit of fat-free cotton in the stem, into a glass stoppered test tube graduated at 10.0 ml.

4. Cool and dilute carefully to volume with chloroform.

5. Carry 10.0 ml. of the cholesterol standard through the determination in the same way.

6. Immerse standard and unknown cholesterol solutions in a water bath at 25°C . for 10 minutes.

7. Add to each, 3.0 ml. of acetic anhydride-sulfuric acid reagent, stopper and mix. Replace in water bath and read at exactly 45 minutes after the addition of the reagent.

8. Set the photometer at 100 with chloroform (not water). Transfer the colored solution to a photometer tube and read using filter 640 (or 620) or a spectrophotometer set at 625 mu. (Care should be taken to avoid introduction of water.)

Cholesterol Esters:

1. Into a 50 ml. beaker, place about 10 mg. of digitonin and dissolve with heating in 1-2 ml. of alcohol, (or use 1 ml. of 1.0% alcoholic digitonin).

2. Add with a pipet 15 ml. of alcohol-ether extract of serum (prepared as above). Mix with the digitonin solution. Evaporate just to dryness using a gentle air current and gentle heat. Do not overheat. Place the flask under the infra-red lamp for ten minutes.

3. Add 15 ml. petroleum ether, cover with a watch glass. Boil on hot plate. (No flames near! No sparking contact!) until half the volume remains. Pour through cotton into a 100 ml. beaker as in total cholesterol and re-extract with 8 ml., boil to 1/2 volume and filter, repeat with 6 ml. then with 4 ml. and again with 4 ml. (15, 8, 6, 4, 4) Some workers prefer using (15, 12, 10, 8, 6).

4. Evaporate the extracts just to dryness, heat under infra-red lamp 10 min. and proceed as for total cholesterol, extracting repeatedly, using chloroform (3, 2, 2, 2, 2).

5. Proceed with dilution, cooling, color development, etc. as in total cholesterol using the same standard.

Calculation:

Total Cholesterol: $\frac{D_u}{D_s} \times 0.8 \times \frac{100}{0.4} = \text{mg. cholesterol/100 ml. serum.}$

or

$\frac{D_u}{D_s} \times 200 = \text{mg. \%}$ or $\frac{200}{D_s} \times D_u = \text{mg. \%}$

Cholesterol Esters: $\frac{D_u}{D_s} \times 0.8 \times \frac{100}{0.6} = \text{mg. cholesterol esters/100 ml. serum}$

$\frac{D_u}{D_s} \times 133 = \text{mg. \% cholesterol esters.}$

Interpretation:

Normal values of total cholesterol range from 150-250 mg.%. Normal esters are 60-75% of total.

Total cholesterol is increased in diabetes mellitus with lipemia, nephritis, obstruction of bile ducts. % ester decreases in liver diseases. In thyroid diseases cholesterol varies inversely with the B.M.R.

Method II (References 3 and 4)

Principle:

Serum proteins are precipitated and lipids are extracted by means of an alcohol-acetone mixture. Free cholesterol is precipitated by the use of digitonin without saponification and the total cholesterol is precipitated by digitonin after saponification. The separated and washed digitonides are then determined by the Liebermann-Burchard reaction, after being dissolved in glacial acetic acid.

Reagents:

1. Acetone-alcohol mixture. Mix equal volumes of acetone and 95% ethyl alcohol.
2. Acetone-ether mixture. Mix 1 volume of acetone with 2 volumes of ether (anesthesia ether may be used).
3. Ethyl ether-analytical reagent or anesthesia ether may be used.
4. Glacial acetic acid.
5. Digitonin, 0.5% solution in 50% ethanol. Dissolve 500 mg. of digitonin in 100 ml. of the alcohol at 60° C. 50% ethanol is prepared by mixing 55 ml. of 95% alcohol and 45 ml. of distilled water.

6. Potassium hydroxide 33%. Dissolve 10 g. KOH in 20 ml. distilled water. Store in a bottle with a dropping pipet with rubber bulb.
7. Phenolphthalein. 1% in 95% ethanol.
8. Acetic acid 10%. Dilute 10 ml. of glacial acetic acid to 100 ml. with water.
9. Concentrated sulfuric acid-analytical grade.
10. Acetic anhydride.
11. Acetic anhydride-sulfuric acid reagent. Place an amount of acetic anhydride sufficient for the number of samples to be read in a glass-stoppered flask and chill in an ice bath. With the flask still in the ice bath, add concentrated sulfuric acid in the proportion of 1 ml. to 20 ml. of the acetic anhydride with agitation. Insert the stopper, and remove the flask from the bath, shake vigorously for a few moments and return to the ice bath.
12. Cholesterol stock standard. Dissolve 100 mg. (analytical balance) of cholesterol in glacial acetic acid and make up to 100 ml. with acid.
13. Cholesterol working standard. Dilute 10 ml. of the stock standard with glacial acetic acid up to 100 ml. This contains 0.1 mg. cholesterol per ml. of solution.

Procedure:

Lipid Extraction and Deproteinization.

1. Add 1.00 ml. of serum, plasma, or blood, dropwise, with constant shaking, to about 10 ml. of alcohol-acetone mixture in a 25-ml. volumetric flask, glass-stoppered.
2. Heat carefully in a boiling water bath, with shaking, just to the boiling point. Remove from the boiling water bath, but continue shaking for 30 to 60 seconds.
3. Cool to room temperature, make up to volume with alcohol-acetone mixture. Insert the stopper and mix thoroughly.
4. Filter through a fat-free filter paper (Whatman #43) into a large dry test tube. Keep the funnel covered with a watch-glass to avoid loss by evaporation.

Free Cholesterol Isolation

1. Pipet 7.00 ml. of the filtrate into a heavy-wall conical centrifuge tube. Add 3.5 ml. of digitonin solution and 2 drops of acetic acid 10% and stir well with a thin glass rod. Leave the rod in the tube and allow to stand in a closed jar overnight, at room temperature.
2. In the morning, transfer the tube to a rack, stir the contents gently to free precipitate adhering to the wall near the surface of the liquid. Remove the rod and "file" it.
3. Centrifuge the tube at high speed (2500 rpm) for 10-15 minutes to pack the precipitate so that the supernatant can be decanted without loss of solid material.
4. Decant the supernatant with a slow steady motion watching closely to ensure that no loss of solid material occurs. If any loss occurs the sample must be discarded and the determination repeated.
5. Drain the tube in a vertical position for a few moments, remove the last drop by a clean cloth or paper. Return the rod to the tube, wash down the wall of the tube and the rod with 5 ml. of alcohol-acetone mixture and stir the precipitate thoroughly. Remove and "file" the rod and centrifuge the tube for 5 minutes. Decant the supernatant.

6. Wash twice more in the same manner with ether. Return the rod to the tube. The tube and its contents may be stored for several days at this stage. If color development is to be carried out immediately, the ether should be removed from the precipitate by placing the tube in a moderately warm water bath for a few minutes.

Precipitation of Total Cholesterol

1. Pipet 3.00 ml. of filtrate into a heavy-wall conical centrifuge tube. Add 3 drops of 33% KOH and stir with a thin glass rod until all droplets of the alkali have disappeared especially at the tip of the tube.

2. Incubate at 38°C. for 30 minutes. Add alcohol-acetone to the 5 ml. mark, add 1 drop of phenolphthalein and titrate with acetic acid 10% until the pink color disappears. Add one more drop of acetic acid and 3.0 ml. of digitonin solution, stir well with the glass rod and allow to stand overnight as above under free cholesterol.

3. The sample is washed as above under free cholesterol except that the precipitate is washed with ether only once.

Development and Reading of the Color

1. Add 2.00 ml. glacial acetic acid and dissolve by the aid of heat (boiling water bath) and stir with the glass rod.

2. Prepare similar tubes for standard and blank by pipetting 2.00 ml. of glacial acetic acid (blank) and 2.00 ml. of cholesterol working standard (standard) into conical centrifuge tubes (heavy walled).

3. Transfer the tubes to a water bath at 25°C. out of contact with direct sunlight. When the tubes are at 25°C. add to each in succession 4.00 ml. of the acetic anhydride-sulfuric acid mixture, mix well with the glass rod and stand in the 25°C. water bath for exactly 30 minutes.

4. Adjust the photometer to 100% transmittance at a wavelength of 625 mμ with the blank tube and read each tube in order 30-31 minutes after the addition of the color reagent. The same photometer tube may be used for each reading without rinsing if the tube is well drained between readings.

Calculation:

A. Free Cholesterol

$$(D_u/D_s) \times 0.2 \times 100/(7/25) = \text{mg. free cholesterol per 100 ml. serum.}$$

$$(D_u/D_s) \times 71.4 = \text{mg. free cholesterol per 100 ml. serum.}$$

B. Total Cholesterol

$$(D_u/D_s) \times 0.2 \times 100/(3/25) = \text{mg. total cholesterol per 100 ml. serum}$$

$$(D_u/D_s) \times 166.7 = \text{mg. total cholesterol/100 ml. serum}$$

Note:

If the amount of serum is limited, the following modification may be used: About 2 ml. of acetone-ethanol are placed in a graduated conical heavy-walled centrifuge tube and 0.2 ml. of serum is added slowly in such a way that it runs down the wall of the flask and forms a layer under the solvent. The contents are immediately mixed thoroughly with a swirling motion. The solvent is brought just to the boil on a steam bath, and the tube is then cooled, and acetone-ethanol added to the 5.00 ml. mark. The suspension is thoroughly mixed and filtered into a small test tube through a fat-free filter. Two ml. of the filtrate are taken for free cholesterol and 1 ml. is taken for total cholesterol with the other reagents reduced approximately in proportion but with the technique remaining the same. Color development and reading are carried out as described above.

Allowable variations in the procedure (Reference 4):

1. The proportion of serum volume to extract volume may be decreased to compensate for exceptionally high cholesterol concentrations, but not increased.
2. The volume of extract in which precipitation is carried out may be varied at will, provided that 1 drop of alkali solution per 1 ml. of the extract is added in the determination of total cholesterol, and that 1 ml. of digitonin solution is added for each 2 ml. of acetone-ethanol.
3. The volume in which color is developed may be varied without reference to the volume in which precipitation was carried out, provided that 2 ml. of the acetic anhydride-sulfuric acid reagent are added for each 1 ml. of acetic acid.

A simple rapid procedure for the determination of total cholesterol is given by Carr and Dreker in Reference 5. It however, offers little or no advantage over the method of Schoenheimer and Sperry for the determination of free cholesterol.

Purification of Petroleum Ether - Procedure:

1. Petroleum ether must stand over H_2SO_4 for at least two weeks.
2. Pour petroleum ether from bottle (being extremely careful not to get any acid over) and neutralize using 10% NaOH. Check pH with red and blue litmus paper. Discard acid.
3. Siphon off the layer of NaOH (bottom).
4. Wash petroleum ether 3 times with distilled water, siphoning off H_2O each time.
5. Dry with approximately 200 g. Na_2CO_3 .
6. Filter through 12 inch filter paper into flask for redistillation.
7. Connect apparatus and redistill, using hot plate not flame.
8. Discard first 50-100 ml.
9. Check temperature during distillation. 30-60°C.

CONGO RED TEST

Serum

Reference:

Paunz, L., Magyar Orvosi Arch. 25, 499 (1924). Chem. Abstr. 19, 1009 (1925)

Principle:

In amyloidosis (deposition in various tissues of a protein giving a reddish iodine test) congo red rapidly disappears from the blood. In this condition 60-100 % will disappear in one hour; normally 15-30% disappears. Amyloidosis occurs in patients suffering from a long-standing suppurative (pus-forming) process and also idiopathically.

Apparatus:

Colorimeter or Photometer.

Reagents:

1. 1% Congo red in water (sterile for injection)
2. Acetone

Procedure:

1. Inject intravenously for each 50 kg. of body weight, 6 ml. of 1.0% congo red. Four minutes later collect 10 ml. of blood; 1 hr. after injection collect 10 ml. of blood.
2. Allow blood to clot, centrifuge, remove the serum.
3. To a measured volume of serum add 2 volumes of acetone. Shake well and centrifuge while stoppered.
4. Remove supernatant fluids and compare in colorimeter, or measure optical density against a blank of 67% acetone.

Calculation:

A. Colorimetric

$$\frac{\text{Depth of 4 minute specimen}}{\text{Depth of 1 hour specimen}} \times 100 = \% \text{ dye remaining in the plasma}$$

$$100 - \text{per cent in plasma} = \% \text{ absorbed}$$

B. Photometric: (515 mμ)

$$\frac{D \text{ 1 hour}}{D \text{ 4 min.}} \times 100 = \% \text{ dye retained in plasma}$$

$$\text{Example: } \frac{0.052}{0.204} \times 100 = 25\% \text{ retention}$$

$$100 - 25 = 75\% \text{ absorbed}$$

Interpretation:

See principle. The urine may contain large amounts of the dye in a lipoid nephrosis.

CREATININE (and CREATINE) - Serum and Urine

References:

1. Taussky, H.H., J. Biol. Chem., **208**, 853 (1954)
2. Clark, L.C., and Thompson, H.L., Anal. Chem. **21**, 1218 (1949)
3. Løken, F., Scand. J. Clin. Lab. Invest. **6**, 325 (1954)
4. Van Pilsum, J.F., and Bovis, M., Clin. Chem. **3**, 90 (1957)

Principle:

Creatinine reacts with alkaline picrate solution to give a red color - the so-called Jaffè reaction. This reaction is not specific for creatinine and other substances in blood, especially in the red blood cell also give a red color. However, the total chromogen expressed as creatinine is commonly used for clinical purposes, and a simple determination for both creatinine and its precursor creatine is given in Method I. Method II is more specific for creatinine and is recommended for the determination of creatinine clearances.

Method I (References 1 and 2)

Reagents:

1. Picric acid 0.040 N. Weigh out 9.16 g. of high grade analytical reagent picric acid, dissolve with stirring in about 900 ml. distilled water and dilute to 1000 ml.
2. Sodium hydroxide 0.75 N. Dilute 30 ml. of 2.5 N NaOH up to 100 ml. with distilled water.
3. Creatinine stock standard. Dissolve 1.0000 g. pure dry creatinine (not the zinc salt) in 0.1 N HCl and make up to 1000 ml. with the acid. This solution is stable almost indefinitely. 1.610 g. creatinine - ZnCl_2 may be used.
4. Creatinine working standard. Dilute 1.00 ml. of stock standard up to 100 ml. with water. Mix well. Each ml. contains 0.01 mg. creatinine.
5. Sodium tungstate, 5%. Dissolve 5.0 g. $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ in water and make up to 100 ml.
6. Sulfuric acid 0.67 N. See p. 30.

Procedure:

Urine:

1. Dilute 5.00 ml. of urine to 500 ml. in a volumetric flask. Mix well.
2. Pipet 6.00 ml. of the diluted urine into a test tube and add 2.00 ml. of 0.04 N picric acid and 2.00 ml. of 0.75 N NaOH. Mix well.
3. Treat 6.00 ml. of distilled water and 6.00 ml. of standard creatinine (working) in the same way.
4. Allow to stand for 15 minutes and read photometrically within the next 30 minutes, using 515 mu.

Serum

1. Dilute 2.00 ml. of plasma or serum with 2.00 ml. of distilled water, add 2.00 ml. of 5% sodium tungstate and 2.00 ml. of 0.67 N sulfuric acid, adding the latter dropwise with shaking. Allow to stand for ten minutes, shake again and filter.

2. Pipet 3.00 ml. of filtrate into a test tube, add 3.00 ml. of distilled water. Then add 2.00 ml. of picric acid 0.04 N and 2.00 ml. 0.75 N NaOH. Mix well. Allow to stand 15 minutes and compare photometrically with a blank consisting of 2.00 ml. of distilled water and a standard consisting of 2.00 ml. of creatinine standard carried through all steps as given above (including Step 1), using 515 mμ.

Calculation:

Urine:

$$(D_u/D_s) \times 0.06 \times 100/0.06 = \text{mg. creatinine/100 ml. urine}$$

$$(D_u/D_s) \times 1000 = \text{mg. creatinine per liter of urine.}$$

Serum:

$$(D_u/D_s) \times 0.02 \times 100/2 = \text{mg. creatinine/100 ml. serum}$$

$$(D_u/D_s) = \text{mg. creatinine/100 ml. serum}$$

Method II (References 3 and 4)

Principle:

A trichloroacetic acid protein-free filtrate of serum is treated with Lloyd's reagent (a form of hydrated aluminum silicate) which adsorbs the creatinine, but not other picrate chromogens of serum. The creatinine is then eluted and the color developed by alkaline picrate solution.

Reagents:

1. Trichloroacetic acid 1 N. Weigh out 16.3 g. reagent grade $\text{CCl}_3\cdot\text{COOH}$ in a beaker, add 100 ml. distilled water and dissolve without heat.

2. Lloyd's reagent - each new bottle should be tested for its ability to adsorb creatinine.

3. 0.04 N picric acid. A saturated solution of picric acid is prepared by adding about 16 g. of picric acid to one liter of water in a 2 L. flask. When heated to about 80°C. complete solution takes place. Cool to room temperature (some acid will crystallize out). Dilute about 690 ml. of this saturated solution to one liter and titrate with 0.1 N NaOH using phenolphthalein as an indicator. The strength is then adjusted to between 0.0395 N and 0.0405 N.

4. Sodium hydroxide 0.15 N. Dilute 6 ml. of 2.5 N NaOH (see solutions p. 28) up to 100 ml. with distilled water. Titrate with standard HCl solution and adjust to between 0.148 N to 0.152 N.

5. Alkaline picrate reagent. Just before use, mix 40.0 ml. of 0.04 N picric acid and 60.0 ml. of 0.15 N NaOH.

6. Creatinine-stock standard. Dissolve 1.0000 g. pure dry creatinine in approximately 0.1 N HCl and dilute to the mark in a 1 liter volumetric flask using the same acid. 1.610 g. creatinine-ZnCl₂ may be used.

7. Creatinine-working standard. Dilute 2.00 ml. of stock standard to 100 ml. with water. Mix well. 3.5 ml. contains 0.07 mg. (70 ug.). 5 ug./ml. will be the concentration in the final colored solution.

Procedure:

A. Deproteinization. To 3.5 ml. serum add 3.5 ml. H₂O and 7.0 ml. 1 N CCl₃·COOH. Stopper. Shake vigorously, and allow to stand 10 minutes, again shake vigorously, and centrifuge for 10 minutes or until the precipitate is well packed. Treat 3.5 ml. of water and 3.5 ml. of dilute working standard similarly at the same time.

B. Adsorption. To a test tube containing 200 mg. Lloyd's reagent transfer 10 ml. of supernatant (by use of cotton-tipped pipet). Stopper, and gently mix the contents of the tube once each minute for 10 minutes. Centrifuge and decant the supernatant and allow to drain 3-4 minutes. Discard the supernatant.

C. Elution and color development. Add 10.0 ml. of the alkaline picrate reagent, mix thoroughly by shaking, and gently mix once each 2 minutes for 10 minutes. Centrifuge and transfer sufficient of the supernatant for photometric comparison to cuvetts (using a pipet). After another 30 minutes compare photometrically against the water blank and the standard using a spectrophotometer set at 515 mu.

Urine:

Dilute 5.00 ml. urine to 500 ml. in a volumetric flask. Mix well. Follow procedure as given under serum above, steps A., B., and C.

Calculation:

Serum:

$$(D_u/D_s) \times 0.05 \times 100/2.5 = \text{mg. creatinine/100 ml. serum}$$

$$(D_u/D_s) \times 2 = \text{mg. creatinine/100 ml. serum}$$

Urine:

$$(D_u/D_s) \times 0.05 \times 100/0.025 = \text{mg. creatinine/100 ml. urine}$$

$$(D_u/D_s) \times 200 = \text{mg. creatinine/100 ml. urine}$$

CREATINE Urine and Serum

Principle:

Creatine in the urine is converted to creatinine by heating in the presence of acid. After heating the total creatinine is determined as above. The difference between total creatinine and pre-formed creatinine gives the value for creatine.

Reagents:

As for the determination of creatinine (Method I).

Procedure:

Urine:

1. Pipet 6.00 ml. of diluted (1:100) urine into a test tube calibrated at 10.00 ml.
2. Add 1.00 ml. 0.04 N picric acid.
3. Place the test tube in a boiling water bath for forty-five minutes.
4. At the same time treat a blank consisting of 6.00 ml. of distilled water and a standard consisting of 6.00 ml. of working standard in the same way.
5. Cool to room temperature after forty-five minutes. Add another 1.00 ml. picric acid to each tube and mix well. Then add 2.00 ml. 0.75 N NaOH, add distilled water to the 10 ml. mark, mix well. Allow to stand for 15 minutes and read within the next 30 minutes.

Serum:

Treat a mixture of 3.00 ml. of 1:4 acid filtrate (see Method I) and 3.00 ml. of distilled water as outlined above.

Calculation:

As under Creatinine (Method I)

Total creatinine - preformed creatinine = creatine (as creatinine).

Creatine (as creatinine) $\times 1.159$ = creatine

For a somewhat more elaborate procedure for eliminating non-specific chromogen interference in both urine and serum see Tausky, H. H., Clinica Chimica Acta, 1, 210 (1956).

Interpretation:

Urine:

Creatine phosphate in the muscle serves as a reservoir for high energy phosphate. During muscle contraction creatine-phosphate is broken down to creatine and phosphate. Creatinine is derived from creatine at a very steady rate which is uninfluenced by most metabolic changes. Creatinine is a waste product and is found in the urine in amounts which are very constant from day to day for any given individual. The normal daily urinary excretion is between 1 and 2 grams, higher in men than in women.

The normal creatine excretion is very low or completely absent, except in children and in women in pregnancy and for a short time after delivery. Tausky (1) found from 0 to 500 mg. creatine excreted per 24 hours.

Creatinine excretion changes very little with disease, except in very severe kidney dysfunction.

Creatine excretion may increase in muscular disorder such as myasthenia gravis, muscular dystrophies, etc. Any rapid decrease in the muscle mass may contribute to an elevated urinary creatine.

Serum:

By Method I., serum creatinine values between 1 and 2 mg. per 100 ml. are found. By Method II., rather less, 0.7 to 1.4 mg./100 ml. are found. This is a reflection of the fact that some of the Jaffe positive material in plasma is not creatinine but substances giving the same color. In the red blood cell, almost half of the chromogenic material is non-creatinine in nature.

Most of the creatine is found in the cells; whole blood creatine being from 2 to 7 mg. % normally; and plasma creatine is well under 1 mg. %.

Plasma creatinine increases in renal disease, somewhat later than does urea. More than 5 mg. % in a chronic nephritis indicates a poor prognosis. Acute renal syndromes may show much higher creatinine values and still have a good recovery. Plasma creatine determinations have been little used. There is some evidence that creatine levels are increased in hyperthyroidism.

Creatinine clearance studies have been widely used as a measure of glomerular filtration rate. In dogs inulin and creatinine clearance studies give identical results. In man, with normal plasma creatinine levels, this is approximately true, but with elevated plasma levels, the creatinine clearance does not represent glomerular filtration alone, since some tubular excretion is taking place.

ESTERASE (TRIBUTYRINASE)

Serum

References:

1. Goldstein, N. P., Epstein, J. H., and Roe, J. H., J. Lab. & Clin. Med. **33**, 1047 (1948).
2. Alper, C., Standard Methods of Clinical Chemistry **1**, 71 (1953).
3. See also references under Lipase - Serum

Principle:

Esterase of serum is allowed to catalyze the hydrolysis of the ester bond in tributyrin (glyceryl tributyrate). The acid liberated is quantitatively estimated by titration with standard alkali.

Reagents:

1. Calcium acetate solution 2%.
2. Sodium diethylbarbiturate solution 0.5%.
3. Sodium choleate, solid.
4. Tween 20.
5. Methocel (15 centipoise)
6. Tributyrin, c.p. This may be purified by neutralizing with sodium bicarbonate, washing with distilled water and distilling under reduced pressure.
7. Esterase substrate.
Add 250 ml. 2% (w/v) calcium acetate solution to 250 ml. 0.5% (w/v) sodium diethylbarbiturate solution. Add to this mixture, 6.7 mg. sodium choleate, while beating in the Waring blender. Add 0.25 ml. Tween 20, and 0.5 g. Methocel. Continue beating and add 6.25 ml. tributyrin dropwise from a buret. Mix by beating 3.5 minutes. Adjust by NaOH to pH 8.55. Store in refrigerator. This emulsion should be good for at least 14 days.
8. Ethanol-ether 9:1. Add 100 ml. of a diethyl ether to 900 ml. of 95% ethanol. Mix well.
9. Alkali 0.05 N. Either sodium or potassium hydroxide may be used.

Procedure:

1. Measure 20 ml. portions of esterase substrate into each of two large test tubes. Warm the tubes to 37°C. in a water bath.
2. Add 1.0 ml. of serum to one tube, mix well and incubate both tubes at 37°C. for 1 hour.
3. At the end of the 1-hour incubation period, add 1.0 ml. of serum to the control tube and immediately pour the contents of each tube into a 100 ml. beaker, and transfer completely by rinsing each tube with 50 ml. of 9:1 alcohol ether mixture. Mix well with a glass stirring rod.
4. Titrate the mixture with 0.05 N alkali to a distinct pink with phenolphthalein as the indicator. If a pH meter is used, titrate to pH 10.65.

Calculation:

See Lipase, p. 208.

Interpretation:

There are present in human sera a number of enzymes capable of splitting the ester bond which have been classified as follows:

1. Cholinesterases

a. True acetylcholinesterase

This enzyme is found in large quantities in brain and in erythrocytes. It is inhibited by relatively low concentration of eserine and by relatively low concentration of substrate.

b. Pseudo-cholinesterases

These are found in tissue and in serum. They are characterized by the high concentration of eserine and of choline-ester substrate required for inhibition. The esterase measured by the procedure described above falls into this class.

2. All-esterases (simple esterase)

These are enzymes catalyzing the hydrolysis of esters of low molecular weight fatty acids and differing from pseudo-cholinesterases in that they are not inhibited by eserine.

3. Lipases

These are enzymes catalyzing the hydrolysis of esters of high molecular weight fatty acids - such as olive oil, etc.

4. Sterol esterases

These are enzymes catalyzing the hydrolysis of (e.g.) cholesterol esters.

The variations with disease of these various types of esterases have not been well studied. The following gives a general outline of the data obtained.

1. Pancreatic disease - In acute exacerbations of chronic relapsing pancreatitis, in acute pancreatitis, and in obstructive pancreatic duct lesions, the lipase is elevated, the esterase is normal.

2. Liver disease - In some types of toxic liver disease there may be decreased serum amylase and lipase but increased pseudocholinesterase. In moderate and severe cirrhosis, the levels are below normal.

ESTROGENS

Urine

References:

- Anker, Rudolph M.: Determination of estrogens in stored urines of pregnancy, *J. Clin. Endo. & Metab.* **15**, 210-214 (1955).
- Bachman, Carl, Pettit, Dorothy S.: Photometric determination of estrogens. III. A procedure for the estimation of the estrogens of pregnancy urine, *J. Biol. Chem.* **138**, 689-704 (1941).

Principle:

A 100 ml. aliquot of a 24-hour urine specimen is taken for assay. The determination comprises (a) hydrolysis of the water-soluble conjugates in urine; (b) extraction of the steroid moieties and their separation by their partition between solvents; and (c) spectrophotometric estimation of the color developed by sulfuric acid-ethanol reagent.

Reagents:

1. Ethyl ether: absolute, peroxide-free.
2. Hydrochloric acid: concentrated, reagent grade.
3. 9% sodium bicarbonate: 9 g. reagent grade NaHCO_3 in 91 ml. of distilled water.
4. 9% sodium carbonate: 9 g. of reagent grade, anhydrous, sodium carbonate in 91 ml. of distilled water.
5. Benzene: reagent grade.
6. 1 N sodium hydroxide: 20 g. of the reagent grade pellets of NaOH are dissolved in 500 ml. of distilled water.
7. 45% sulfuric acid: 90 ml. concentrated, reagent grade acid is carefully added to 110 ml. of distilled water and cooled to room temperature.
8. Ethanol: absolute, dehydrated, reagent of NF grade.
9. Color reagent A: Carefully and slowly add 80 ml. of concentrated sulfuric acid to 20 ml. absolute ethanol. Cool to room temperature and store in refrigerator. This reagent is good for 14 days.
10. Color reagent B: carefully add 60 ml. of concentrated sulfuric acid to 30 ml. of absolute ethanol. Cool to room temperature and store in refrigerator. This reagent is good for 14 days.
11. Standard estradiol: 10 mg. of pure estradiol in 100 ml. of 95% ethanol.

Procedure:

1. The total volume of the specimen (preserved with 15 ml. of toluene) is recorded. Carefully measure out a 100-ml. aliquot of the specimen using a graduated cylinder.
2. Place the aliquot in a 250 ml. Erlenmeyer flask with two glass beads to prevent bumping, add 15 ml. hydrochloric acid. Bring the contents of the flask to a boil, and continue to boil GENTLY for 10 minutes. When boiling is completed, cover neck of flask with a 50 ml. beaker, and chill contents of flask to below 15°C . in the refrigerator.
3. Extract the hydrolyzed urine in a 250 ml. separatory funnel with one 100 ml., and two 50 ml. portions of ether. Combine ether extracts; discard extracted urine.

4. Wash the ether extract once with 8 ml. of sodium bicarbonate, in the separatory funnel. Allow to settle five minutes. Discard aqueous phase.

5. Evaporate the ether extract to dryness in a 250 ml. glass evaporating dish in a hood. Avoid flames. Dissolve residue in 0.5 ml. of ethanol and add 35 ml. of benzene.

6. Extract the benzene solution with one 35 ml. and two 17 ml. portions of 9% sodium carbonate and one 5 ml. portion of water. The benzene contains the hormones estrone and estradiol. The aqueous phase contains the hormone, estriol.

Estriol Phase

1. Acidify the aqueous phase from the above step to litmus paper using hydrochloric acid.

2. Extract the acidified solution with three 40 ml. portions of ether. Discard aqueous phase; combine ether extracts.

3. Wash ether extract with 12 ml. sodium bicarbonate; discard the aqueous phase. Evaporate ether extract to dryness.

4. Dissolve residue in 0.5 ml. ethanol, dilute with 35 ml. of benzene. Wash solution once with 1 ml. of sodium bicarbonate and discard washing.

5. The estriol is transferred to water by washing the benzene with three 50 ml. portions of water. The combined water extracts are evaporated to dryness and the residue taken up in 2 ml. of ethanol for final assay.

Estrone-Estradiol Phase

1. The benzene solution from Step 6 is washed with 10 ml. of 45% sulfuric acid followed by two 20 ml. portions of water. The washings are discarded.

2. The estrogens are extracted from the benzene with four 35 ml. portions of 1 N sodium hydroxide. The benzene is now discarded. Make the alkaline extract acid to litmus paper using hydrochloric acid.

3. Extract the acidified sodium hydroxide with one 100 ml. and two 50 ml. portions of ether. Combine the ether extracts; discard the aqueous phase.

4. Wash the ether extract with 15 ml. of 45% sulfuric acid. Next wash with two 25 ml. portions of 9% sodium carbonate, followed by two final washings with 25 ml. of water. Discard all washings. Evaporate ether extract to dryness in a 250 ml. glass evaporating dish.

5. The residue is taken up in two ml. of ethanol, and stored in a stoppered cylinder until final assay.

Color Development

1. The alcoholic urine extracts from both the Estriol and Estradiol-Estrone fractions are placed in test tubes appropriately marked. The full 2.0 ml. volume of alcoholic urine extract is used in each case.

2. 0.25 ml. of alcoholic estradiol standard is placed in a third test tube and appropriately marked.

3. Evaporate the contents of all tubes to dryness in either a vacuum desiccator or a gentle stream of air.

4. To each tube add 2.0 ml. of Color Reagent A. Stopper each tube loosely to

exclude moisture. Heat in a boiling water bath for 10 minutes, shaking well at two minute intervals. The color, which is quite stable, will develop at this point. Cool all tubes to room temperature.

5. Quantitatively transfer the contents of each tube to 10 ml. volumetric flasks. Dilute to the mark with color reagent B. Mix thoroughly.

6. The optical density is determined at 406 mu., 456 mu., and 505 mu. on the Beckman Spectrophotometer using 1 cm. quartz cells against 3 ml. of color reagent B as the blank.

Calculations:

To calculate the amount of estrogen present, a correction for extraneous non-specific color must first be made using the following equation:

$$(1) \quad C.D. (456) = OD_{(456)} - \left(\frac{OD_{(406)} + OD_{(506)}}{2} \right)$$

The corrected optical density at 456 mu. (CD_{456}) is substituted in the following equation to find the amount of Estrogen present in the 100 ml. aliquot of urine used.

$$(2) \quad \frac{CD_{456}}{E_f} = \text{micrograms \% (ug \%)}$$

The factor E_f in the above equation varies with the fraction under determination, and represents the optical density for 1 microgram of the estrogen diluted to 10 ml. as is done in the outlined procedure.

	E_f
Estriol Fraction	- 0.013
Estrone-Estradiol Fraction	- 0.018
Standard Estradiol	- 0.009

In calculating the 25 ug standard the results should not vary more than from 24-26 micrograms. If a greater or lesser result is obtained, Color Reagents A and B probably have become too decomposed, and fresh solutions should be prepared with the procedure repeated over from the beginning.

Lastly, the total amount of Estrogen in the 24-hour specimen is calculated:

$$(3) \quad \frac{\text{ug \%}}{100} \times \text{total volume} = \text{total Estrogen (in micrograms)}$$

The results are reported on Standard Form 514a in the following manner:

Total volume	_____ ml.
Estriol	_____ micrograms
Estrone-Estradiol	_____ micrograms (calculated as estrone)
Total Estrogen	_____ micrograms

Example: A 24-hour specimen of urine of total volume 1000 ml. gave the following readings in optical density on the Beckman Spectrophotometer.

Fraction	456 mμ	406 mμ	506 mμ
Estrone-Estradiol	0.615	0.801	0.169
Estriol	0.567	0.973	0.073
Standard Estradiol	0.305	0.125	0.051

The results were calculated in the following manner:

Estriol Fraction

$$(1) CD_{456} = 0.567 - \frac{0.973 \times 0.073}{2}$$

$$CD_{456} = 0.567 - 0.523 = 0.044$$

$$(2) \frac{0.044}{0.013} = 3.4$$

$$(3) \frac{3.4}{100} \times 1000 = 34 \text{ micrograms}$$

Estrone-Estradiol Fraction:

$$(1) CD_{456} = 0.615 - 0.801 \times 0.169$$

$$CD_{456} = 0.615 - 0.485 = 0.130$$

$$(2) \frac{0.130}{0.018} = 7.2$$

$$(3) \frac{7.2}{100} \times 1000 = 72 \text{ micrograms}$$

Standard Estradiol:

$$(1) CD_{456} = 0.305 - \frac{0.125 \times 0.051}{2}$$

$$= 0.305 - 0.088 = 0.217$$

$$(2) \frac{0.217}{0.009} = 24.1 \text{ micrograms}$$

Results:

Total volume	1000	ml.
Estriol	34	micrograms
Estrone-Estradiol	74	micrograms (cal. as estrone)
Total Estrogen	108	micrograms

Notes:

This procedure is primarily designed for estrogen assay of urines of late pregnancy or urines in which a high amount of estrogen excretion is suspected. Normal male and female urines usually fail to give results above zero because of excessive chromogenic interference. In calculating the results, equation #1 will usually give a negative value in these cases which does not necessarily indicate the absence of estrogen. Satisfactory results will usually be obtained, however, when the total 24-hour estrogen excretion is 100 micrograms or higher.

In reporting the results of assay of normal urines, when Equation #1 gives a negative value, the results of analysis may be reported as "Total Estrogen content does not appear to exceed the normal limits."

Interpretation:

Normal Values:

Female

Normal Adult before Menopause
Normal Adult after Menopause
Pregnancy

**Total Estrogen in
micrograms per day**

- 20 to 80
- below 50
- *see note

Male

Normal Adult

- 0 to 5

*During pregnancy the total estrogen rises to about 12 to 40 milligrams per day, with estriol comprising about 90% of the total amount. Estriol is usually absent in normal female urines.

1000	Total volume
34	Estriol
44	Estro-ne-Estrial
108	Total Estrogen
micrograms	
micrograms (cal. as estro-ne)	

FAT IN FECES

References:

Hepler, O. E. *Manual of Clinical Laboratory Methods*, Springfield, Ill. Charles C. Thomas. 4th Ed. (1949).

Fowweather, F. S. and W. N. Anderson. A method for the determination of fat in feces. *Biochem. J.* **40**, 350-351 (1946).

Van de Karver, J. H., Nainink, H. Ten and Weyers, H. A., *J. Biol Chem.* **177**, 347 (1949).

Principle:

Total fat includes free fatty acids, soaps, and neutral fat fractions. Hydrochloric acid is added to a portion of feces to convert the soaps to fatty acids. The total fat is then removed in an ether extraction, purified by means of petroleum ether, and determined gravimetrically. Another portion of feces (not treated with hydrochloric acid) is extracted with ether and the amount of free fatty acids and neutral fats are determined gravimetrically. From this precipitate the free fatty acids are dissolved in benzene and titrated with 0.1 N sodium alcoholate.

Reagents:

1. Concentrated hydrochloric acid.
2. Anhydrous ethyl ether.
3. 95 per cent ethyl alcohol.
4. Petroleum ether - boiling point should be below 60° C.
5. Benzene.
6. 0.1 N sodium alcoholate
 - a. Place about 500 ml. absolute ethyl alcohol (redistilled) in a 1 liter volumetric flask.
 - b. Add 2.3 grams freshly cut metallic sodium; when dissolved, dilute to volume with alcohol. Keep away from flames.
 - c. Titrate with 0.1 N hydrochloric acid using 2 drops 0.5 per cent alcoholic phenolphthalein as indicator.
 - d. Adjust normality to 0.1 with alcohol as the solvent.

Procedure:

1. Record total weight of fresh specimen.
2. Thoroughly mix and homogenize specimen to afford even distribution of contents.
3. Weigh onto two previously tared aluminum dishes about 2-3 grams feces from specimen. Label 1 and 2.
4. Place dishes on hot plate in hood until all odor is dispelled. Then continue heating in drying oven (95 to 100° C.) until samples are at constant weight. From final weighing calculate DRY WEIGHT and MOISTURE CONTENT of specimen.
5. Weigh two samples of about 2-3 grams fresh specimen into two 250 ml. centrifuge bottles. Label A and B.
6. To bottle A add 3 ml. concentrated hydrochloric acid and 30 ml. distilled water. Bottle A is used for determination of TOTAL FAT.

7. To bottle B add 30 ml. distilled water. Bottle B is used for the determination of FATTY ACIDS and NEUTRAL FAT.

8. Add 20 ml. anhydrous ethyl ether to each bottle and shake for five minutes. (Use caution to avoid loss of ether.) Cool under tap if necessary. Let stand 5 minutes.

9. Add 17 ml. 95 per cent ethyl alcohol to bottle A and 20 ml. to bottle B. Mix contents by a quick rotary motion and cool to room temperature in running water.

10. Stopper bottles and shake vigorously for 5 minutes. Then centrifuge at 2000 rpm.

11. Transfer the ether layer, as completely as possible, into 150 ml. beakers. (Label A and B). Repeat extraction twice using 20 ml. portions of ether. Combine the three extractions.

12. Wash the stopper and sides of the bottles with three successive 5 ml. portions of ether and add each to the ether extractions.

13. Evaporate the combined extractions and washings to dryness.

14. Add 20 ml. petroleum ether to each beaker, warm on a water bath, and filter through fat-free filter paper into two previously tared 100 ml. beakers. Repeat twice using 10 ml. portions of petroleum ether. Label A and B.

15. Evaporate the petroleum ether to dryness. Dry residue to constant weight in a 37° C. oven. Record weights.

16. Dissolve residue in beaker B in 50 ml. benzene and heat almost to boiling.

17. Titrate while still hot with 0.1 N sodium alcoholate, using two drops alcoholic phenolphthalein as indicator. Titrate until color no longer deepens.

Calculations:

1. Dry Matter:
$$\frac{\text{dried weight sample 1}}{\text{wet weight sample 1}} \times 100 = \text{per cent dry matter}$$

(Repeat with sample 2 and take the average)

2. Total Fat:

a. Calculate the weight of dry matter in sample A by multiplying the wet weight by the per cent of dry matter found.

b.
$$\frac{\text{total fat (sample A)}}{\text{dry weight (sample A)}} \times 100 = \text{per cent total fat}$$

3. Free fatty acids plus neutral fat:

a. Calculate the weight of dry matter in sample B by multiplying the wet weight by the per cent of dry matter found.

b.
$$\frac{\text{free fatty acids plus neutral fat (sample B)}}{\text{dry weight (sample B)}} \times 100 \text{ equals per cent free}$$

fatty acids and neutral fat.

4. Soaps:

a. (Per cent total fat) minus (per cent free fatty acids plus neutral fat) equals per cent soaps.

5. Free fatty acids:

- a. 1 ml. of 0.1 N sodium alcoholate titrates 28.2 mgm. of oleic acid or 28.4 mgm. of stearic acid (average 28.3).
- b. The number of mls. of 0.1 N sodium alcoholate used in titration times 28.3 equals mgm. of free fatty acids in sample B.
- c. Convert to grams and

$$\frac{\text{weight of free fatty acids (sample B)}}{\text{dry weight (sample B)}} \times 100 = \text{per cent free fatty acids}$$

6. Neutral fat:

- a. (Per cent free fatty acids plus neutral fat) minus (per cent free fatty acids) equals per cent neutral fat.

7. Consolidate report as follows:

Total weight of specimen	grams
Moisture	per cent
Dry weight of specimen	grams

FAT CONTENT (based on dry weight)

Soaps (calculated as Oleic

and Stearic acid) per cent

Free fatty acids per cent

Total fatty acids per cent

Neutral fat per cent

Total fat per cent

Notes:

1. The analysis should be made on fresh feces as fat decreases on standing even when specimen is frozen.
2. Blank determinations should be run to rule out any fatty substances in the reagents.
3. The amount of dry matter, and likewise the moisture content, is extremely variable in the normal stool. Values should be expressed in terms of dry matter to be significant.
4. Drying alters the chemical composition of feces so the lipids are determined in "wet" feces but reported as per cent of "dry" weight of feces.

Interpretation:

1. Normal values:

- a. Dry matter: 4.6 - 38 per cent
- Total fat: 7.3 - 27.6 per cent
- Free fatty acids: 1.05 - 10 per cent
- Soaps as fatty acids: 0.54 - 11.4 per cent
- Neutral: 2.49 - 11.8 per cent
- Neutral fat as per cent of total fat: 24.5 - 60.1 per cent

2. Abnormal values

- a. Cellac disease and obstructive jaundice: increase in total fat, soaps and

free fatty acids; neutral fat is normal.

b. Pancreatic deficiency: Increase in total fat, neutral fat and soaps with normal or low fatty acids,

c. Nontropical sprue: Increase in total fat, fatty acids and neutral fat.

d. Tropical sprue: Increase in total fat and fatty acids, and decrease in neutral fat.

e. Gastroenteritis: Increase in all fat fractions.

FAT CONTENT (based on dry weight)	
Soaps (calculated as oleic and stearic acid)	per cent
Free fatty acids	per cent
Total fatty acids	per cent
Neutral fat	per cent
Total fat	per cent
Dry weight of specimen	
Moisture	
Total weight of specimen	

- Notes:**
1. The analysis should be made on fresh feces as fat decreases on standing even when specimen is frozen.
 2. Blank determinations should be run to rule out any fatty substances in the reagents.
 3. The amount of dry matter, and likewise the moisture content, is extremely variable in the normal stool. Values should be expressed in terms of dry matter to be significant.
 4. Drying alters the chemical composition of feces so the lipids are determined in "wet" feces but reported as per cent of "dry" weight of feces.

Interpretation:

1. Normal values:
 - a. Dry matter: 4.8 - 35 per cent
 - b. Total fat: 7.0 - 27.0 per cent
 - c. Free fatty acids: 1.05 - 10 per cent
 - d. Soaps as fatty acids: 0.54 - 11.4 per cent
 - e. Neutral: 2.48 - 11.8 per cent
 - f. Neutral fat as per cent of total fat: 24.0 - 80.1 per cent
2. Abnormal values:
 - a. Certain diseases and obstructive jaundice: increase in total fat, soaps and

FIBRINOGEN
Plasma
(Turbidimetric)

Reference:

- Fowell, A. H., American J. Clinical Pathology, 25, 340 (1955)
Parfentjev, I. A., et al., Arch. Biochem. 46, 470 (1953)

Principle:

Fibrinogen is a globulin more easily precipitated than the other plasma globulins. It is practically quantitatively precipitated by 12% $(\text{NH}_4)_2\text{SO}_4$. The plasma is mixed with an appropriate salt solution and the resulting turbidity is read in a photometer against the same plasma diluted in 0.85% saline. The fibrinogen is calculated by means of an equation determined empirically using purified fibrinogen diluted with fibrinogen free serum. Since fibrinogen does have some solubility in the reagent, a simple factor cannot be used, --see Notes below.

Reagents:

1. 0.85% NaCl; Dissolve 8.50 g. NaCl (reagent grade) up to one liter with distilled water.
2. Precipitant solution: To 133.33 g. reagent grade ammonium sulfate and 10.0 g. NaCl, add 0.025 g. Merthiolate to suppress microbial growth. The volume is brought to one liter with distilled water and sufficient 10 N NaOH added to bring the pH to 7 (using 0.02% phenol red as an outside indicator).
3. Serum (for standardization): Pooled normal human serum (from well coagulated blood specimens).
4. Fibrinogen (purified) with clottable protein assay. Warner Chilcott brand was used to calibrate this procedure.

Procedure:

Plasma for this determination is collected preferably in sodium citrate but oxalate or EDTA (Ethylenediaminetetraacetic acid), di-sodium salt, may be used. To 0.50 ml. of plasma (carefully separated and free of erythrocytes), in a photometer tube (22 x 200 mm.), is added 6.00 ml. of precipitant solution. For a blank add to 0.50 ml. of the same plasma 6.00 ml. of 0.85% saline. After three minutes compare photometrically setting the blank at 100 using (515 mμ).

Calculation:

Read the percentage fibrinogen from a graph derived from the standardization procedure given below. Note that the line does not pass through 0, 0.

Standardization

1. Dissolve gently a vial of fibrinogen containing 6 mg. clottable protein (fibrinogen) in exactly 2.00 ml. of fibrinogen free serum.
2. Into three photometer cuvetts add as follows, using serum and the above reconstituted plasma (serum and fibrinogen):

Tube	Fibrinogen %	ml. Serum	ml. Reconst. Plasma	ml. Precipitant Solution
1.	0.00	1.00	0.00	12.00
2.	0.075	0.75	0.25	12.00
3.	0.150	0.50	0.50	12.00
4.	0.30	0.00	1.00	12.00

Mix each tube immediately after the addition of the precipitant and after exactly three minutes, mix and compare photometrically using 515 mu.

Notes:

1. Fibrinogen levels lower than a certain value (approximately 0.06%) cannot be measured by this method.

2. If flocculation has occurred at the time of reading give the sample a vigorous shake to resuspend the fibrinogen.

Interpretation:

Normal levels of fibrinogen range from 0.113-0.380% averaging 0.246%.

Elevated values are seen in most acute infections and may be correlated with increased sedimentation rates.

Decreases may be seen in very severe hepatic deficiencies or in congenital defects in synthetic metabolism, intravascular clotting, generalized, or in localized large scale fibrin formation.

FOLLICLE STIMULATING HORMONE - Bioassay Urine

References:

Dekanski, J., The kaolin-adsorption method for the quantitative assay of urinary gonadotrophins. Brit. J. Exp. Path. 30, 272-82 (1949).

Crooke, A.C., W.R. Butt, J.D. Ingram, and L.E. Romanchuck, Chemical assay of gonadotrophin in urine. The Lancet. 1, 379-83 (1954).

Principle:

Since there is no specific chemical reaction by which gonadotrophins can be measured, the process described is essentially an extractive process using kaolin as an adsorbant of the gonadotrophins and subsequent re-precipitation of the extract. An eluted extract is then assayed by its activity in mice.

Apparatus:

For each determination, four immature female white mice are needed.

Reagents:

1. Glacial acetic acid
2. Hydrion or alkali-acid paper
3. Sodium chloride
4. Ethyl alcohol 95%
5. Kaolin 20% (w/v) aqueous suspension
6. Ammonium hydroxide 1N. (67.5 ml. diluted to 1 liter)

Procedure:

1. The total volume of the urine specimen is poured into a large graduated cylinder and measured. If more than one specimen is run at one time, the volumes are made equal by an addition of distilled water.
2. Adjust the pH of the urine to 4.5 with glacial acetic acid. Use either pHYdrion or alk-acid paper.
3. Add 5 ml. of 20% Kaolin suspension for each 100 ml. of specimen. (Equalizing the volumes allows equal amounts of kaolin to be added to each specimen). Add 1-2 g. of sodium chloride and allow to settle in the refrigerator for about one hour.
4. Siphon off the supernatant liquid being careful not to disturb the settled kaolin.
5. Transfer the kaolin to a 250 ml. centrifuge bottle with distilled water and centrifuge at 1500 rpm for about 10 minutes. Decant and discard the supernatant liquid.
6. 1 N NH_4OH is added to the residue in the bottles in a volume which equals the volume of the original kaolin suspension added. The precipitate is stirred well into the NH_4OH with a stirring rod which is removed and saved.
7. Centrifuge and decant the supernatant into a beaker.
8. Repeat step #6. and combine the supernatants.
9. Centrifuge the combined supernatants. Discard the precipitate and adjust the pH of the liquid to 8.5 with glacial acetic acid using indicator paper.
10. Centrifuge and discard the precipitate formed at pH 8.5. Reduce pH to 5.5 with

acetic acid. Add a volume of 95% ethyl alcohol (or acetone) which is equal to four times the volume of the acidified solution.

11. Place in a glass stoppered Erlenmeyer flask and allow to stand overnight in the refrigerator.

12. Centrifuge the entire sample using the same centrifuge bottle over and over discarding the supernatant after each centrifugation.

13. Place the bottle in a vacuum dessicator and evacuate until dry.

14. When the protein in the bottle is dry (usually overnight) add 7.5 ml. of distilled water. Stir well by swirling and then centrifuge. Take off the supernatant with a drop-per pipet and dilute it as follows:

(1)	4 ml. extract / 1 ml. of H ₂ O	- 3 Mouse Units Level
(2)	1.67 ml. extract / 3.33 ml. H ₂ O	- 9 " " "
(3)	0.58 ml. extract / 4.42 ml. H ₂ O	-27 " " "
(4)	0.19 ml. extract / 4.81 ml. H ₂ O	-81 " " "

15. Inject each twice daily with 0.5 ml. of each dilution for two days. Inject mice once on third day and sacrifice on the fourth day. The weakest dilution at which the uterus and Fallopian tube are twice the normal size gives the titer of the extract.

Notes:

The urine sample must consist of a 24-hour specimen and if shipped from a distance greater than 24 hours traveling time, 15 ml. of chloroform may be added as a preservative.

Interpretation:

The normal male and female give negative tests except as indicated.

Tests may be positive in

Males with

Testicular hypofunction

Seminoma or teratoma of testes

Females in

Pregnancy

Menopause

Ovarian hypofunction

Chorionepithelioma

Hydatidiform mole

and other disease, primary and secondary of the pituitary gland.

GLUCOSE

Blood and Spinal Fluid

Reference:

Nelson, Norton: J. Biol. Chem., **153**, 375-380 (1944) modified and adapted.

Principle:

The glucose in a blood filtrate is oxidized by cupric ion (Cu^{++}) in an alkaline medium, and the cuprous oxide produced is then reacted with acid arsenomolybdate to produce a solution of blue molybdenum oxides whose concentration is directly proportional to the amount of glucose present in the sample. Blood for glucose determinations should be collected in fluoride tubes to avoid the loss of glucose by bacterial and erythrocyte glycolysis.

Reagents:

1. Deproteinizing solutions: see under PROTEIN-FREE FILTRATES for the principles involved and solutions required.
2. Alkaline Copper reagent - for colorimetric technique.
 - 28 g. Na_2HPO_4 anhydrous
 - 100 ml. 1 N NaOH or 40 ml. 2.5 N NaOH
 - 40 g. $\text{KNaC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ (sodium potassium tartrate)
 - 8 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 - 180 g. Na_2SO_4 anhydrousDissolve phosphate and tartrate in 700 ml. of water; add the NaOH; then add 80 ml. of 10% CuSO_4 ; then add the sodium sulfate, stir until dissolved. Dilute to 1 L. Let stand 1-2 days, decant and filter.
3. Arsenomolybdate color reagent:
 - Dissolve 25 g. of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ (ammonium paramolybdate) in 450 ml. of water. Add 21 ml. of concentrated H_2SO_4 and mix. Dissolve 3 g. of $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ in 25 ml. of water and mix the two solutions. Place in incubator at 37°C . for 24-48 hours.
4. Glucose stock standard:
 - Dissolve exactly 1.000 g. of pure anhydrous glucose in 0.25% benzoic acid, dilute with 0.25% benzoic acid to 100.0 ml. and mix.
5. Glucose dilute working standard:
 - Add exactly 1.00 ml. of stock standard to a 100 ml. volumetric flask, add 0.25% benzoic acid to the mark and mix well. This results in a 10 mg.% glucose solution. Prepare fresh each week.
6. 0.25% benzoic acid solution:
 - In a 2 liter volumetric flask, dissolve 5.0 g. benzoic acid. Mix well.

Procedure:

1. Pipet 1.00 ml. of a protein-free filtrate into a 25 ml. Folin-Wu sugar tube.
2. Prepare a blank using 1.0 ml. distilled water and a standard by using 1.00 ml. of dilute (10 mg./100 ml.) glucose standard.

3. To each of the three above in a 25.0 ml. Folin-Wu tube add 1.0 ml. mixed upper reagent; mix by shaking.
4. Cover with aluminum foil caps or glass marbles and immerse simultaneously in a boiling water bath, for 10 minutes.
5. Cool for three minutes in a pan of cold water.
6. Add 1.0 ml. of arseno-molybdate reagent to each tube, mix and dissolve the cuprous oxide by shaking.
7. Let stand 10 minutes and dilute to the 25 ml. mark and mix by several inversions.
8. Read in the photometer (515 mu) using the blank to set 100.

Calculations:

$$(D_u/D_s) \times C_s \times (100/0.1) = C_u = (D_u/D_s) \times 100 \text{ (mg./100 ml. blood)}$$

Example:

$$T_u = 30.2; D_u = 0.520 \quad C_s = 0.1 \text{ mg.}$$

$$T_s = 26.8 \quad D_s = 0.572$$

$$(0.520/0.572) \times 0.1 \times (100/0.1) = (0.520/0.572) \times 100 = 91 \text{ mg.}\%$$

Precautions:

1. Be sure boiling water bath is really boiling.
2. Time boiling accurately.
3. Mix well each time as directed.

Interpretation:

Normal blood sugars for Folin-Wu tungstic acid filtrates are between 80 and 120 mg./100 ml.; for Zn(OH)_2 filtrates 60-90 mg.%. True fermentable blood sugar is 60-80 mg.%. Increases are seen after ingestion of food, in diabetes and in emotional stress. Decreases are seen in insulin overdosage and with active islet cell adenomas of the pancreas.

Spinal Fluid:

1. Dilute the spinal fluid 1:5 (1 \neq 4) with water and use as a protein-free filtrate. Divide nominal results by 2.
2. If the sample contains protein, deproteinize by Ba(OH)_2 - ZnSO_4 method and proceed as above.

GLUCOSE TOLERANCE

References:

1. Anderson, G. E., et al., Am. J. Clin. Nutrition 4, 673 (1956)
2. Silverstone, F.A., M. Brandfonbrener, W.W. Shock and M.J. Ylengst, J. Clin. Invest., 36, 504-14 (1957)
3. Smith, L.E. and Shock, N.W., J. Gerontology 4, 27 (1949)

This test determines the ability of the various organs concerned to handle a rapid influx of glucose. The organs involved include the pancreas (islet cells), the liver, the pituitary, the adrenal cortex, and possibly others. It is of great value in detecting early diabetes.

Procedure:

The patient should have been on an average diet for the preceding two or three days. He should have fasted for the 12 or 14 hours immediately preceding the test. He can be allowed, and should be encouraged, to drink water enough not to be dehydrated and to have an adequate urine flow.

1. Have patient void. Save specimen, Take a blood sample for glucose determination.
2. Have patient drink a solution of 50 g. glucose dissolved in 300 to 400 ml. of water and flavored with the juice of 1/4 to 1/2 a lemon. Some techniques use doses of one gram per kilogram of body weight.
3. Take blood specimens for glucose determination at 1/2, 1, 2, and 3 hours after the ingestion of the glucose. Collect urine specimens at approximately the same time.

Determine the glucose concentrations in the five blood specimens and test the urine specimens qualitatively for glucose. Plot the results.

Interpretation:

Blood: There are four criteria which should be considered in the interpretation of an oral glucose tolerance curve.

	<u>Normal Values</u>
1. The fasting blood glucose value.	90-120
2. The highest blood sugar value.	130-140
3. Time at which the highest value was reached.	45-60 min.
4. Time of return to the fasting level.	1 1/2-2 1/2 hrs.

Urine: With a normal renal threshold, not more than a very small amount of glucose should appear in the urine. When glucose does appear it gives a rough indica-

tion of the renal threshold when simultaneous blood sugars are taken into consideration. All urine samples giving a positive qualitative test for glucose are pooled and the total glucose determined.

Intravenous glucose tolerance test:

This test is carried out whenever altered intestinal absorption may require it. In the great majority of cases, the oral test is quite adequate.

Procedure:

One suggested method is as follows:

1. The patient is prepared as outlined above for the oral glucose tolerance test.
2. Fasting blood and urine samples are taken.
3. 0.5 g. glucose per kilogram of body weight are given intravenously in the form of a 20% solution over a 5-minute period.

A fixed amount of glucose may be used with little effect on the results.

4. Blood specimens are collected from the opposite arm at the end of the injection and at the end of the one-hour, two-hour, and three-hour periods following the start of the injection.

Interpretation:

1. Fasting blood glucose value
2. Original level (or lower) reached in

Normal Value

90-120 (Folin-Wu)
30-60 minutes

Insulin Tolerance Test:

This test is used to investigate patients with various endocrine disorders.

Patient preparation: A diet containing more than 300 g. of carbohydrate daily must be taken by the patient for 3 days preceding the test. The test is carried out on the fourth day with the patient in a fasting state.

Procedure:

1. Blood is taken for a control fasting blood sugar.
2. Insulin is injected intravenously (0.1 units per kilogram body weight).
3. Additional blood samples are taken for blood sugar at 20, 30, 45, 60, 90 and 120 minutes after the insulin injection.

Interpretation:

1. The normal response is an immediate fall (at about 30 minutes) to about 40% of the fasting level, followed by a gradual increase back up to normal fasting values within 90-120 minutes. There are two abnormal types of response.

a. Insulin Resistance: This is evidenced by a very slight and delayed fall in blood sugar values. This is seen in some cases of: Diabetes mellitus; Adrenal cortical hyperfunction (Cushings syndrome); Anterior pituitary hyperfunction (acromegaly).

b. Hypoglycemic Atonia: This is evidenced by a normal fall in blood sugar but the subsequent rise does not occur or is delayed. This is seen in some cases of: Anterior pituitary hypofunction (Simmond's disease); Adrenal cortical hypofunction (Addison's disease); Hyperinsulinism.

5-HYDROXY-INDOLEACETIC ACID

Urine (Qualitative)

References:

Sjoerdsma, A., Weissbach, H., & Udenfriend, S: Simple test for diagnosis of metastatic carcinoid. *J.A.M.A.* **159**, 397 (1955).

Udenfriend, S., Weissbach, H., & Clark, C.: The estimation of 5-hydroxy-tryptamine (serotonin) in biological tissues. *J. Biol. Chem.* **215**, 337-344 (1955).

Udenfriend, S., Weissbach, H., & Titus, E.: The identification of 5-hydroxy-3-indoleacetic acid in normal urine, and a method for its assay. *J. Biol. Chem.* **216**, 499-506 (1955).

Principle:

1-nitroso-2-naphthol in the presence of a small amount of nitrous acid will yield a purple derivative with 5-hydroxy-indoles and this reaction is the basis for detection of abnormally large amounts of 5-hydroxy-indoleacetic acid in urine (40 mg. or more per 24-hour specimen). False positives are given by p-hydroxyacetanilide which is found in the urine only after administration of acetanilide drugs. Color formation is sometimes inhibited by large amounts of keto acids.

Reagents:

1. 1-nitroso-2-naphthol (Eastman) - 100 mg. of the compound is dissolved in 100 ml. of 95% ethanol.
2. Reagent A - 2.5 g. of pure sodium nitrite is dissolved in 100 ml. of distilled water. Keep solution refrigerated. Prepare fresh every 20 days.
3. Reagent B - 19.6 g. of sulfuric acid (concentrated reagent grade) is weighed in a 50 ml. beaker. Add the acid to 85 ml. of distilled water.
4. Ethylene dichloride - purified.

Procedure:

1. Into a 5-inch test tube measure 0.2 ml. of the urine to be tested. Mark this tube "T". Into a second 5-inch test tube, marked "C" for control, measure 0.2 ml. of a known negative urine.
2. Add 0.8 ml. distilled water to each tube.
3. Prepare the Diazo reagent by adding 0.2 ml. of reagent A to 5 ml. of reagent B. This solution must be used within thirty minutes.
4. To both test tubes add 0.5 ml. of 1-nitroso-2-naphthol solution and mix well.
5. To both tubes add 0.5 ml. of Diazo reagent (prepared in step 3), and again mix well. Allow solutions to stand for five minutes.
6. Add 5 ml. of ethylene dichloride to each tube and mix well to extract the excess 1-nitroso-2-naphthol. If turbidity results, the tubes should be centrifuged. A purple to black color in the upper layer is positive for 5-hydroxy-indoleacetic acid. A yellow color which often appears is of no significance. A positive test indicates the presence of over 8 mg. per liter.

Interpretation:

A positive test for 5-hydroxy-indoleacetic acid is comparatively rare. The normal

24-hour output is between 2 to 8 mg. When positive, this test is a specific indication of a metastatic carcinoid condition. In 5 patients with carcinoid syndrome the 24-hour output varied from 140 to 628 mg.

References:

1. Spoorbans, A., Weissbach, H., & Udenfriend, S.: Simple test for diagnosis of metastatic carcinoid. *J. A. M. A.* 158, 387 (1955).
2. Udenfriend, S., Weissbach, H., & Clark, C.: The estimation of 5-hydroxytryptamine (serotonin) in biological tissues. *J. Biol. Chem.* 215, 337-344 (1955).
3. Udenfriend, S., Weissbach, H., & Tins, E.: The identification of 5-hydroxy-3-indoleacetic acid in normal urine, and a method for its assay. *J. Biol. Chem.* 216, 493-506 (1955).

Principle:

1-nitroso-2-naphthol in the presence of a small amount of nitrous acid will yield a purple derivative with 5-hydroxy-indoles and this reaction is the basis for detection of abnormally large amounts of 5-hydroxy-indoleacetic acid in urine (40 mg. or more per 24-hour specimen). False positives are given by p-hydroxyacetanilide which is found in the urine only after administration of acetanilide drugs. Color formation is sometimes inhibited by large amounts of keto acids.

Reagents:

1. 1-nitroso-2-naphthol (Eastman) - 100 mg. of the compound is dissolved in 100 ml. of 95% ethanol.
2. Reagent A - 2.5 g. of pure sodium nitrite is dissolved in 100 ml. of distilled water. Keep solution refrigerated. Prepare fresh every 30 days.
3. Reagent B - 10.5 g. of sulfuric acid (concentrated reagent grade) is weighed in a 50 ml. beaker. Add the acid to 85 ml. of distilled water.
4. Ethylene dichloride - purified.

Procedure:

- A. Into a 5-inch test tube measure 0.2 ml. of the urine to be tested. Mark this tube "T". Into a second 5-inch test tube, marked "C" for control, measure 0.2 ml. of a known negative urine.
1. Add 0.5 ml. distilled water to each tube.
2. Prepare the Dixco reagent by adding 0.2 ml. of reagent A to 5 ml. of reagent B. This solution must be used within thirty minutes.
3. To both test tubes add 0.5 ml. of 1-nitroso-2-naphthol solution and mix well.
4. To both tubes add 0.5 ml. of Dixco reagent (prepared in step 2), and again mix well. Allow solutions to stand for five minutes.
5. Add 5 ml. of ethylene dichloride to each tube and mix well to extract the excess 1-nitroso-2-naphthol. If turbidity results, the tubes should be centrifuged. A purple to black color in the upper layer is positive for 5-hydroxy-indoleacetic acid. A yellow color which often appears is of no significance. A positive test indicates the presence of over 8 mg. per liter.

Interpretation:

A positive test for 5-hydroxy-indoleacetic acid is comparatively rare. The normal

ICTERUS INDEX

Serum

Principle:

The intensity of the yellow color of serum, usually due to bilirubin, is determined by comparison with a standard potassium dichromate solution.

Reagents:

5% sodium citrate solution for diluting serum.

$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ (U.S.P.) 50 g. dissolve in H_2O and dilute to 100 ml. Mix.

Standards:

Weigh out 1.000 g. of $\text{K}_2\text{Cr}_2\text{O}_7$, dissolve in water, add 2 drops of concentrated H_2SO_4 , dilute up to 100 ml., and mix. Into clean, dry test tubes place respectively 0.1, 0.3, 0.5, 0.7, 1.0, 1.2, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 ml. of this solution. Make each volume up to 10.0 ml. with water, and mix. Select test tubes, 11 x 100 mm. size, Pyrex, that have uniform diameters, and clean and dry tubes. Fill about two-thirds full with the above solutions, and seal off. Label each tube according to the number of tenths of a ml. of the 1% dichromate solution used in making it, so that the tubes are labeled from 1 to 100 icterus units. Keep these standards in the dark while not being used.

Method:

Use selected tubes like the above, and of a diameter uniform with the above. Place 1 ml. of serum without hemolysis in one of these test tubes, and compare with the series of standards. From this preliminary comparison, dilute the serum with 5% sodium citrate (if necessary) so that the final comparison is made with an intensity of 15 units or less. Taking the dilution into account, calculate the ICTERUS INDEX of the original serum.

Remarks:

The ICTERUS INDEX gives the number of times the serum would have to be diluted in order for it to match a 1 to 10,000 solution of potassium dichromate (0.01% $\text{K}_2\text{Cr}_2\text{O}_7$). The normal value is 3 to 5 units. Icterus or jaundice is seldom visible in the skin or sclera of a patient until the index rises to about 15 units. The level at which the skin changes color is largely dependent on the amount of diffusible bilirubin present, while the non-diffusible form has little effect on skin color.

Even traces of hemoglobin make it difficult or impossible to obtain a match with the standards. Lipemia also interferes; so the patient should be in a fasting condition. Occasionally carotenemia contributes to the amount of yellow color in the serum.

IODINE PROTEIN-BOUND Serum

References

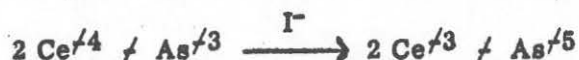
1. Thompson, H. L., Klugerman, M. R. and Truemper, J., "A method for protein-bound iodine: the kinetics and the use of controls in the ashing technique," J. Lab. and Clin. Med., 47, 149-63 (1946).
2. Barker, S. B., Humphrey, M. J. and Soley, M. H., "The clinical determination of protein-bound iodine," J. Clin. Invest., 30, 55-62 (1951)
3. Klugerman, M. R., "A simple and rapid calculation in Barker's method for blood-protein-bound iodine," Am. J. Clin. Path., 24, 490-495 (1954).

Principle:

The estimation of serum protein-bound iodine (PBI) has become an important tool in confirming the existence of thyroid dysfunction. Since its introduction the PBI determination has been proven to be a more reliable and reproducible indicator of thyroid function than the basal metabolic rate (BMR) although it, too, has its own pitfalls.

In the dry-ashing sodium carbonate method of PBI determination, the iodine is isolated from the serum by precipitation and washing of protein followed by drying and incineration. These steps eliminate constituents that would interfere with the colorimetric determination of iodine. The iodine concentration in the ash is then determined.

The colorimetric technique for the determination of trace quantities of iodine is based on the catalytic action of iodide on the ceric-arsenite system.



A definite linear relationship between reaction time, i.e., the rate of decolorization of the ceric ion, and the iodide concentration was shown. Therefore, by determining the ceric ion concentration at the end of a given period of time the iodide concentration may be calculated. The ceric ion concentration is determined photometrically.

Apparatus:

1. Photoelectric colorimeter: Any of the standard instruments may be used for the PBI determination if a proper range has been ascertained by estimating the optimum concentration of ceric ion for the ceric-iodide-arsenite system.

2. Cuvets

3. Muffle furnace
4. Drying oven
5. Heavy wall glass ignition tubes (25 x 200 mm.)

Reagents

1. Zinc Sulfate: dissolve 100 grams $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in water and dilute to one liter.
2. Sodium Hydroxide (0.5N): Dissolve 20 grams NaOH in water and dilute to one liter. Note: The zinc sulfate and sodium hydroxide solutions should be so adjusted that when 10.0 ml. of the zinc sulfate is diluted with 50 to 70 ml. H_2O and titrated with 0.5N NaOH, about 10.8–11.2 ml. of the NaOH will be required to produce a faint permanent pink color with phenolphthalein.
3. Hydrochloric acid (2.0 N): Dilute 170 ml. analytical reagent grade concentrated HCl to one liter with H_2O . Titrate against 1.0 N NaOH.
4. Sulfuric acid (7N): Dilute 200 ml. concentrated H_2SO_4 to one liter with H_2O .
5. Sodium Carbonate (4.0 N): Dissolve 212 grams of Na_2CO_3 (analytical reagent) in H_2O and dilute to one liter. Titrate against standard 1.0 N HCl with methyl orange indicator.
6. Ceric Sulfate (0.012 N): Dissolve 8.02 grams of ceric ammonium sulfate, $(\text{Ce}(\text{SO}_4)_2 \cdot 2(\text{NH}_4)_2\text{SO}_4 \cdot 4\text{H}_2\text{O})$ in a one-liter flask containing 500 ml. H_2O and 230 ml. 7 N H_2SO_4 . When the solution is clear, dilute to the liter mark with H_2O .
7. Arsenious Acid (0.012 N): In a one liter flask dissolve 5.84 grams of As_2O_3 in 30 ml. of 1 N NaOH, warming to hasten solution. Add 300 ml. H_2O and dilute H_2SO_4 until the solution is slightly acid to litmus. This requires approximately 4.5 ml. of the 7 N H_2SO_4 . Dilute with H_2O to the liter mark.
8. Iodide Stock Standard: Dissolve 118.1 mg. NaI, analytical reagent, or 130.8 mg. KI, in H_2O and dilute to one liter (1 ml. = 100.0 micrograms I⁻). Dilute stock is obtained by diluting 4 ml. of the above stock to one liter with H_2O . (1 ml. = 0.40 ug I⁻). Working Stock is obtained by diluting 10 ml. of dilute stock to 100 ml. with H_2O . (1 ml. = 0.04 micrograms I⁻). All of these solutions have been found to keep indefinitely at refrigerator temperatures. It is preferable, however, to prepare a new working standard every week or two.

Analytical Procedure

A. Precipitation and Washing of Protein

1. Add 10.0 ml. H_2O to each ignition tube.

2. Pipet 1.00 ml. serum into each tube.
3. Add 1.0 ml. of the ZnSO_4 reagent and mix well.
4. Add 1.0 ml. of 0.5 N NaOH and mix thoroughly with a stirring rod.
5. Centrifuge for 10 minutes at 3500 rpm.
6. Pour off and discard the supernatant fluid.
7. Add 10.0 ml. H_2O to each tube and mix.
8. Centrifuge and discard supernatant as above.
9. Wash at least one more time.
10. Add 1.0 ml. of 4.0 N Na_2CO_3 solution. Do not mix.

B. Drying and Incineration

1. Place the ignition tubes in the drying oven (85 to 95°C.) to drive off the water. This usually takes about 12 hours.
2. After drying, place the tubes in the muffle furnace (600 \pm 25°C.) for three hours.

C. Colorimetric Determination

1. Add 2 ml. of 2 N HCl to the ash. Tilt the tubes at a 30° angle so that no material is lost in the effervescence. Allow to stand for 10 minutes.
2. Add 1 ml. 7 N H_2SO_4 and 3 mls. H_2O .
3. Add 0.5 ml. of the arsenious acid solution and mix well by shaking.
4. Add 1 ml. of the ceric sulfate reagent, at 30 second intervals, to the samples being tested, and mix well by shaking.
5. Place each tube in a 37°C. water bath immediately after the addition of the ceric sulfate.
6. Set the photometer at 100% transmittance using a wavelength of about 420 mμ with a distilled water blank and at 0% transmittance with a reference blank (1 ml. ceric sulfate solution in 6.5 mls. H_2O). Recheck these setting several times in alternation.
7. Read the transmittance of the first sample exactly 12 minutes after the addition

of the ceric sulfate solution, pouring the solution from the ignition tubes into the appropriate cuvetts. Continue reading each of the other samples at 30 second intervals corresponding to the time of addition of the ceric sulfate solution, each one being read 12 minutes after the ceric sulfate addition.

TABLE 1

SUMMARY OF PROCEDURE

	Serum Sample	Reagent Blank
1. H ₂ O	10.0 ml.	---
2. Serum	1.0 ml.	---
3. ZnSO ₄	1.0 ml.	---
4. 0.5 N NaOH	1.0 ml.	---
5. Centrifuge and discard supernatant, wash with water 2 times		---
6. 4.0 N Na ₂ CO ₃	1.0 ml.	1.0 ml.
7. Dry in oven and incinerate in muffle furnace		
8. 2 N HCl (let sit 10 minutes)	2.0 ml.	2.0 ml.
9. 7 N H ₂ SO ₄	1.0 ml.	1.0 ml.
10. H ₂ O	3.0 ml.	3.0 ml.
11. Arsenious acid	0.5 ml.	0.5 ml.
12. Ceric Sulfate	1.0 ml.	1.0 ml.

Calculations

A standard curve is determined using the prepared NaI or KI standard solutions. From this curve the iodine concentration at a given transmittance may be determined.

The solutions of varying iodide concentration for the establishment of the standard curve are prepared in the following manner.

1. Add the appropriate amount of NaI or KI standard solution and water to each cuvet (see Table II).

2. Add 1.0 ml. of 4.0 N Na_2CO_3 solution to each tube.

3. Add 2.0 ml. of 2.0 N HCl with the tubes tilted at a 30° angle, and let them sit for 10 minutes.

4. Add 1.0 ml. of 7.0 N H_2SO_4 .

5. Add 0.5 ml. of arsenious acid.

6. Add 1.0 ml. of ceric sulfate solution to each cuvet at 30 second intervals; mix, place the tubes in the water bath; read the light transmission in the same manner as described for samples under Analytical Procedure.

7. The standard curve is determined by plotting per cent transmittance against the iodide concentration, on semi-log paper.

TABLE II

Iodide Concentration (ug/ml)	KI or NaI (ml)	H_2O (ml)
0.00	0.0	2.0
0.02	0.2	1.8
0.04	0.4	1.6
0.06	0.6	1.4
0.08	0.8	1.2
0.10	1.0	1.0
0.12	1.2	0.8
0.14	1.4	0.6
0.16	1.6	0.4
0.18	1.8	0.2
0.20	2.0	0.0

Notes

Standards: A standard should be run with each group of samples to serve as a

check on the method, accuracy and equipment. Either of two types of standard may be used: (1) the serum standard which is a commercially prepared serum of known protein-bound iodine concentration (e.g., Versatol by the Warner-Chilcott Laboratories of New York) or (2) the prepared standards of the NaI and KI standard solutions.

The serum standard is run in the same manner as the serum sample with the exception that the standard serum is used instead of the unknown sample.

When NaI or KI solutions are used as standards the precipitation steps of the analytical procedure are omitted. One-half ml. of the standard working NaI or KI solution is placed in an ignition tube, and one ml. of the Na_2CO_3 solution is added. The tube is then carried through the remaining steps of the analytical procedure along with the group of samples.

Blanks: A reagent blank should also be run with each determination. This consists of adding 1.0 ml. of the Na_2CO_3 solution to a dry ignition tube and carrying the tube through the remaining steps in the analytical procedure along with the group of samples. Any contamination occurring in the determination will be shown by the blank.

Contamination: The reagents and glassware may be very easily contaminated by iodine or mercury (which inhibits the reaction) due to the very small amounts being determined. Scrupulous care should be taken to guard against this possibility. It is recommended that a separate room be used for the PBI determinations.

Distilled water: The water used in this determination should be glass redistilled from an alkaline solution.

Interpretation

The PBI is an important clinical and diagnostic tool when used with knowledge of its applicability and limitations. It is more often compatible with clinical and pathologic evaluation of thyroid disorder than the BMR since it is a direct measurement of circulating calorogenic substance while the BMR is an indirect indicator of thyroid function and may be affected by many non-thyroid hypermetabolic and hypometabolic diseases or conditions, as well as by emotional attitude and the responses and level of external stimuli.

The normal PBI concentration in the human body has been found to be 3.5 to 8.0 ug per 100 ml. The PBI is generally elevated in hyperthyroidism and falls after thyroidectomy or treatment with radioactive iodine or antithyroid drugs. In cretinism or hypothyroidism the PBI is generally below normal. However, in some borderline cases when the patient has hyperthyroidism or hypothyroidism the PBI may be just within the normal range. In thyroid carcinoma and thyroiditis values for the PBI follow no consistent pattern.

Nonthyroid diseases, especially neuroses, psychoses, cardiovascular-renal diseases, non-thyroid endocrine disturbances, blood dyscrasias, extrathyroid malignancy, hepatic and biliary tract diseases, infectious diseases and dermatoses seldom cause any variation in the PBI.

The major difficulty in the determination of the PBI is that it is greatly affected by exogenous iodine in any form. Therefore the thyroid status of the patient cannot be determined when the patient has been receiving iodine medication or any drug or treatment involving iodine or bromine.

Organic iodine-containing compounds known to affect protein-bound iodine determinations include iodine-containing radiopaque dyes used in angiocardiology, cholecystography, urography, bronchography, or myelography; iodinated amebicides (chiniofon, iodochlorhydroxyquin, and diiodohydroxyquin); vaginal suppositories such as Floraquin, which contains diiodohydroxyquin; penicillin G diethylaminoethyl ester hydriodide (Neopenil); and iodothiouracil.

Preparations containing inorganic iodine include Lugol's solution, tincture of iodine and syrup of hydriodic acid. Certain vitamin and cod liver oil preparations may also be rich in iodine, while barium sulfate is said to give rise to elevated protein-bound iodine due to contamination with iodine. Sulfo bromophthalein (Bromsulphalein) though not containing iodine, also causes apparent elevation of plasma protein-bound iodine.

IRON Serum

Reference:

Flister, H. J., "Manual of Standardized Procedures for Spectrophotometric Chemistry." Standard Scientific Supply Co., New York City, N. Y. (1950).

Principle:

A sample of plasma or serum is incubated with hydrochloric acid. The protein is then precipitated and the ferric ion (Fe^{+++}) is reduced to the ferrous state with hydroquinone. The ferrous ion is then reacted with α - α' dipyridyl to form an intense red colored complex (Blau reaction).

Apparatus:

1. Ostwald-Folin pipet accurately calibrated to contain 4.00 ml.
2. Coleman spectrophotometer, with small cuvetts.

Reagents:

1. Water, distilled, iron-free: Redistill distilled water in an all-glass pyrex distillation apparatus until iron-free.

Test for the presence of iron: Treat 10 ml. of water with 1 ml. concentrated hydrochloric acid, 0.1 ml. concentrated nitric acid, 4 ml. 3 N potassium thiocyanate and 2 ml. iso-amyl alcohol. Shake thoroughly and allow the alcohol layer to separate: a colorless alcohol layer indicates the absence of iron. Use this test for all reagents and standards and where indicated in the test procedure.

2. Hydrochloric acid 0.3 N. Add 30 ml. of concentrated hydrochloric acid to 1000 ml. of water.

3. Trichloroacetic acid 20 per cent aqueous. Add 20 g. of trichloroacetic acid to 100 ml. volumetric flask and dilute to mark with water. Mix well.

4. Sodium acetate, saturated aqueous. Place 130 g. of sodium acetate, crystalline ($\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$) into a bottle and add 100 ml. of water. Note: complete saturation is essential, excess crystals should be present.

5. Hydroquinone, 1 per cent aqueous. Dissolve 0.5 g. in 50 ml. of water. This solution must be made fresh each day. Ascorbic acid may be substituted for hydroquinone.

6. α - α' dipyridyl, 0.2 per cent aqueous. Dissolve 200 mg. in 10 ml. of water and dilute to 100 ml. Reagent must be colorless. Discard if colored. Keep in brown glass bottle in refrigerator.

7. Iron Standard:

(a) Iron Stock standard: (1 ml. contains 1 mg. of ferric ion). Thoroughly clean a small piece of iron wire of known iron content (special for standardization, usually 99.85 per cent Fe) with fine emery cloth or paper until shining and free of rust and dirt. (A sample of ingot iron may be used. This may be procured from National Bureau of Standards, Washington, D. C.) Knowing the iron content, weigh out an amount of wire that contains 100 mg. of iron. Into a 250 ml. flask place 10 ml.

of concentrated nitric acid and approximately 40 ml. water. Heat to boiling (gently) and add wire, boil (gently) until completely dissolved and one minute longer, cool and transfer to a 100 ml. volumetric flask, dilute to the mark with water.

(b) Dilute standard: (1 ml. contains 0.01 mg. Fe): Place 1.00 ml. of stock standard in 100 ml. volumetric flask and dilute to the mark with water.

Procedure:

1. Into a 15 ml. centrifuge tube, place:
 - 2 ml. of HCl 0.3 N and 4.00 ml. of serum or plasma (rinsing out pipet several times in the tube.) Mix well, especially noting the tip of the centrifuge tube.
2. Place in water bath for one hour at 37° C.
3. Add 2 ml. of 20% trichloroacetic acid dropwise with shaking.
4. Stopper and shake tube vigorously.
5. Allow tube to stand at room temperature for one hour.
6. Centrifuge at high speed (2000 rpm) for 15 minutes.
7. Into a small cuvet place 4.00 ml. of the clear supernatant fluid.
8. Into another cuvet place in order: (Reagent blank.)
 - 2.00 ml. of water
 - 1.00 ml. 0.3 N HCl
 - 1.00 ml. trichloroacetic acid 20%Mix well
9. To each cuvet add in order:
 - 0.5 ml. saturated sodium acetate
 - 0.3 ml. hydroquinone
 - 1.00 ml. a-a' dipyridyl reagentMix well
10. Allow to stand for one hour at room temperature.
11. Read transmittancy of the sample against the reagent blank set at 100 per cent transmittance at 520 mμ.
12. Read the concentration from the calibration curve or calculate by comparison with a standard run simultaneously.

Calibration

In a series of seven accurately calibrated 10 ml. volumetric flasks place respectively 0, 1, 2, 4, 6, 8, 10 ml. of the dilute standard (0.01 mg./ml.) and dilute to volume with iron-free water.

These standards represent concentrations of iron equivalent to 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mg. per 100 ml. of sample. (Prepare just before use).

Transfer 2 ml. of each calibration standard to a correspondingly marked cuvet.

To each cuvet, add in the order named, mixing after each addition.

1 ml. 0.3 N HCl

1 ml. trichloroacetic acid 20%

0.5 ml. saturated sodium acetate

0.3 ml. hydroquinone

1 ml. a-a' dipyridyl

Allow to stand at room temperature for one hour. Set the zero concentration

standard at 100 per cent transmittance at a wavelength of 520 mμ, and record the transmittancy of the 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 mg. per cent standards. (The transmittancy of the 1.0 mg. standard is approximately 26 per cent.)

Plot the observed values on semi-log graph paper (or convert to optical density and plot on linear paper). (The points fall in a straight line which passes through the origin.)

Use this graph to find the concentrations of unknowns.

Notes:

1. Plasma or serum from blood must be withdrawn during postabsorptive state:

The following precautions should be carefully observed to prevent hemolysis.

(Slight traces are of no consequence but samples showing gross hemolysis cannot be used.)

Withdraw blood with minimum stasis, using absolutely dry syringes and stainless steel needles (gold or platinum needles are preferable) and allow the syringe to fill without exerting suction with the plunger. Remove needle from syringe and allow blood to flow slowly down the side of the collection tube without pressure on the plunger. For plasma analysis use sodium citrate as anti-coagulant. (Fluorides, oxalates and tungstates interfere.) Analysis is preferably made on serum. The plasma or serum must be separated from the red cells within one hour after the collection of the sample.

2. It is advisable to run a standard along with each determination instead of relying upon the constancy of the calibration curve.

Interpretation:

The normal range of values is: 0.08 to 0.18 mg. serum iron per 100 ml. serum.

Increased values are seen in hepatitis, hemochromatosis and pernicious anemia.

Decreased values are seen in the iron deficiency anemias.

IRON-BINDING CAPACITY

Blood

Reference:

Varley, Harold, "Practical Clinical Biochemistry", Interscience Publishers Inc., N. Y., N. Y.; page 324 (1954).

Ventura, S., Determination of the unsaturated iron-binding capacity of serum. J. of Clin. Path. 5, 270-274 (1952).

Principle:

The iron in the plasma is bound to a specific iron-binding component of the beta-globulin, only part of which is saturated in normal plasma. The iron protein complex is red, so that it is possible to measure the unsaturated iron-binding capacity, that is, the amount of iron which can be taken up by this serum globulin. A solution of an iron salt is added to the serum, and the increase in red color is read on the Beckman Spectrophotometer. From this reading the per cent of saturation and the Total Iron Binding Capacity may be found if the results of a Serum Iron determination are also known.

Apparatus:

1. Beckman DU Spectrophotometer with four quartz cuvetts.

Reagents:

1. Iron Standard: 35.2 mg. of reagent grade ferrous ammonium sulfate, $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, and 20 ml. of 1 N acetic acid is diluted to 1000 ml. using iron-free, triple distilled water. Mix well. This gives a standard with a concentration of 5 micrograms of iron per ml. of solution.

2. Sodium Chloride Solution: 0.85 gm. of pure, dry sodium chloride is added to a 100 ml. volumetric flask and diluted to the mark with triple distilled water.

3. Water: The water used in every step of this determination should be triple-distilled and iron-free, preferably given a final distillation in all glass apparatus. Since the color is very sensitive in this test, even trace quantities of iron in the water will profoundly alter results.

Procedure:

1. A 20 to 25 ml. specimen of clotted blood is required; at least 6 ml. of serum is required for the test.

2. Using six test tubes, which have been rinsed at least 5 times with iron-free water, number them B, 1, 2, 3, 4, and 5. Add exactly 1.0 ml. of clear, unhemolyzed serum to each tube.

3. To each tube add exactly 1.5 ml. of sodium chloride solution. Mix thoroughly with serum.

4. Add to the tubes the proper proportions of sodium chloride and iron standard as given in the following table:

<u>Tube</u>	<u>Iron Standard</u>	<u>Sodium Chloride</u>
B	0.0 ml.	1.0 ml.
1	0.2 ml.	0.8 ml.
2	0.4 ml.	0.6 ml.
3	0.6 ml.	0.4 ml.
4	0.8 ml.	0.2 ml.
5	1.0 ml.	0.0 ml.

5. Stir the contents of each tube well with stirring rods. Allow to stand six minutes for full color development.

6. Transfer the solutions to quartz cuvetts for reading on the Beckman DU. Readings are made at 520 mμ using tube B for the blank. The serum has become saturated when the optical density has reached a maximum.

7. The number on the first tube to reach saturation is multiplied by 100 to give the amount of iron in micrograms per cent needed for saturation. When Total Iron-Binding Capacity is requested, this result is added to the results of a serum iron determination on the same sample of serum.

Example: The optical density readings on a given sample of serum were found to be:

1.	-	0.40
2.	-	0.56
3.	-	0.65
4.	-	0.64
5.	-	0.65

Tube #3 is the first tube to reach saturation, hence the number 3 is multiplied by 100.

$$3 \times 100 = 300 \text{ micrograms \% (0.3 mg. \%)}$$

To calculate the total iron-binding capacity, this result is added to the results of a previous serum iron which was found to be 0.16 mg. %. Thus:

$$\begin{array}{r}
 0.16 \text{ (Serum Iron)} \\
 + 0.3 \text{ (Iron-binding Capacity)} \\
 \hline
 0.46 \text{ mg. \% (Total Iron Binding Capacity)}
 \end{array}$$

Interpretation:

Following are accepted normal values for the normal adult male and female, given in mg. per cent.

Sodium Chloride

Normal Male

Normal Female

Iron-Binding
Capacity

0.150 - 0.222
(Aver. 0.205)

0.144 - 0.322
(Aver. 0.194)

% Saturation

30% - 44%
(Aver. 34%)

30% - 44%
(Aver. 33%)

Total Iron-Binding
Capacity

0.254 - 0.432
(Aver. 0.311)

0.224 - 0.415
(Aver. 0.288)

In iron deficiency, while serum iron is lowered, there is an increase above normal in both the unsaturated iron-binding capacity and total carrying capacity of the serum. This is in contrast to infection in which serum iron is similarly reduced, but where the iron-binding capacity and total capacity are reduced as well. A high serum iron and high percentage of saturation of the iron-binding protein is found in refractory anemia, pernicious anemia, hemochromatosis, liver disease, and transfusion hemosiderosis.

Example - The optical density readings on a given sample of serum were found to

0.40	-	1
0.36	-	2
0.33	-	3
0.31	-	4
0.28	-	5

Tube #3 is the first tube to reach saturation, hence the number 3 is multiplied by 100.

$$3 \times 100 = 300 \text{ micrograms } \bar{F} \text{ (0.3 mg. } \bar{F})$$

To calculate the total iron-binding capacity, this result is added to the results of a previous serum from which was found to be 0.16 mg. \bar{F} . Thus:

$$\begin{array}{r} 0.16 \text{ (Serum Iron)} \\ 0.3 \text{ (Iron-binding Capacity)} \\ \hline 0.46 \text{ mg. } \bar{F} \text{ (Total Iron Binding Capacity)} \end{array}$$

Interpretation

Following are accepted normal values for the normal adult male and female, given in mg. per cent.

NEUTRAL 17-KETOSTEROIDS

Urine

References:

(1) Callow, N., Callow, R., Emmens, C.: Colorimetric determination of substances containing the grouping $-CH_2 \cdot CO-$ in urine extracts as an indication of androgen content. *Biochem. J.* **32**, 1312-1331 (1938).

(2) Engstrom, W., Mason, H.: A study of the colorimetric assay of urinary 17-ketosteroids. *Endocrin.* **33**, 229-238 (1943).

Principle:

The 17-ketosteroids are separated from their water soluble conjugates by hydrolysis with concentrated HCl. They are then extracted from the hydrolyzed urine with ethyl ether. The ether extract is washed with sodium hydroxide and distilled water to remove all phenolic and acidic impurities, and finally evaporated to dryness. The residue is taken up in absolute ethanol. An aliquot of this alcoholic urine extract is treated with potassium hydroxide and m-dinitrobenzene to develop a red-green color which is compared with a standard in the spectrophotometer.

Apparatus:

1. Coleman Junior Spectrophotometer, Model 6A

2. 12 matched round cuvettes, 19 x 150 mm.

Reagents: (All chemicals used should be of Reagent Grade)

1. Hydrochloric Acid: concentrated analytical grade

2. Ethyl Ether: peroxide-free

3. 2N Sodium Hydroxide Solution: 80 grams of NaOH pellets are dissolved in 500 ml. distilled water in a 1 liter Pyrex volumetric flask, and diluted to the mark when cool. Preserve in a 1 liter polyethylene bottle.

4. Ethanol: Dehydrated, N. F.

5. m-dinitrobenzene: The Reagent Grade compound should be recrystallized once from ethyl alcohol and carefully dried before using. The purified compound should be stored in a tightly stoppered amber bottle in a cool, dark place. For use in this procedure, 0.50 grams of the purified crystals are dissolved in exactly 25 ml. of dehydrated ethanol to make a 2% alcoholic solution. The solution when stored in an amber bottle at room temperature will keep for about 30 days.

6. 8N Potassium Hydroxide: 45 grams of the Reagent Grade pellets are dissolved in 75 ml. of distilled water and then diluted up to the 100 ml. mark in a volumetric flask. The solution is cooled to 15°C. in the refrigerator and then diluted to the mark. The normality should be checked by titration with a primary standard. The solution should be stored in a polyethylene bottle and the titre of the solution checked and corrected every 30 days.

7. Diluting Alcohol: 400 ml. of 95% alcohol is placed in a 500 ml. graduate and diluted with distilled water to the 500 ml. mark. Store in a glass stoppered bottle.

8. Dehydroisoandrosterone Standard: 25 mg. of the steroid is dissolved in exactly 25 ml. dehydrated ethanol and stored in a glass stoppered bottle in the refrigerator. This is the stock solution with a concentration of 1 mg./ml. The working standard is made by carefully diluting 2 ml. of the stock standard with 6 ml. of dehydrated ethanol in a 10 ml. graduated cylinder and mixing well. This gives a working concentration of 0.25 mg. per ml. The working standard is stored at room temperature. (Pure dehydroisoandrosterone may be purchased from Sigma Chemical Co., 4648 Easton Ave., St. Louis 13, Mo.)

Procedure:

1. Accurately measure and record the total volume of the 24 hour specimen.
2. In a 250 ml. Erlenmeyer flask, place a 100 ml. aliquot of the urine. Add 15 ml. of concentrated hydrochloric acid and two hard glass beads. Place a funnel in the neck of the flask to prevent splattering, and bring the contents of the flask to a boil on a hot plate. Continue to boil GENTLY for exactly ten minutes.
3. Immediately cover the flask with a 50 ml. beaker after removing flask from heat, and place in refrigerator until urine reaches room temperature or below.
4. Place cooled urine in a 250 ml. separatory funnel and extract with one 100 ml. and two 50 ml. portions of ether. Combine the ether extracts. Discard extracted urine.
5. Wash the combined ether extracts with 15 ml. portions of 2 N NaOH until the aqueous phase is colorless (usually three washings is sufficient). Discard washings.
6. Wash the ether extracts with three 15 ml. portions of distilled water. Discard washings.
7. Evaporate the ether extract in a 250 ml. glass evaporating dish to dryness in a rapid stream of air in the hood. Under no circumstances should heat be applied to hasten the evaporation of remaining traces of water.
8. Using a glass rod with a rubber policeman, the residue is taken up quantitatively in exactly 2 ml. of dehydrated alcohol, and stored temporarily in a glass stoppered 5 ml. graduate.

9. A test tube rack is set up with 19 x 150 mm. cuvettes: one cuvette marked "RB" for Reagent blank, one marked "S" for Standard. Two cuvettes are required for each sample to be run: one marked "T" for Test and one marked "U" for urine blank. A Urine Blank is run to correct for extraneous color from urinary chromogens. Using 0.2 ml. pipettes (Serological), add the following reagents to the appropriate cuvettes in the exact order given:

TEST "T" (One for each sample)

0.2 ml. alcoholic urine extract
0.2 ml. 2% m-dinitrobenzene
0.2 ml. 8N KOH

URINE BLANK "U" (One for each sample)

0.2 ml. ethanol
0.2 ml. alcoholic urine extract
0.2 ml. 8N KOH

REAGENT BLANK "RB" (Only one required)

0.2 ml. ethanol
0.2 ml. 2% m-dinitrobenzene
0.2 ml. 8N KOH

STANDARD "S" (Only one required)

0.2 ml. dehydroisoandrosterone standard soln.
0.2 ml. 2% m-dinitrobenzene
0.2 ml. 8N KOH

10. Mix the contents of each cuvette well. Place all tubes in a dark place at room temperature, allowing exactly 20 minutes for color development. At the end of this time add 10 ml. of diluting alcohol to each tube using a 10 ml. volumetric pipet.

11. Read all tubes within 7 minutes in the Coleman Spectrophotometer set at 520 mμ. The instrument is first set to read 100% transmission using the Reagent Blank and the Standard and all Tests "T" read as % transmission against it. Next the instrument is reset at 100% transmission using distilled water, and all Urine Blanks "U" read.

Calculations:

1. Convert all readings in % transmission to their corresponding optical density from an appropriate conversion chart.

2. To calculate the amount of 17-ketosteroids as mg%; Substitute readings in optical density in the following formula:

$$\text{Concentration} = \frac{(\text{OD of Test "T"}) - (\text{OD of Urine Blank "U"})}{(\text{OD of the Standard "S"})} \times 0.5$$

The factor 0.5 in the above equation is derived from the Standard Photometric Equation (p. 57).

3. Total Ketosteroid Content of 24 hour specimen: Substitute appropriate values in the following equation:

$$\frac{\text{c onc. in mg\%}}{100} \times \text{Total Volume in mls.} = \text{mg. per 24 hour volume.}$$

Example: A 24 hour specimen submitted was measured and found to have a Total Volume of 3590 ml. Readings on the Spectrophotometer were found to be:

Test "T" - 73.25%	(OD = 0.1352)
Urine Blank "U" - 97.50%	(OD = 0.0110)
Standard - 48.50%	(OD = 0.3140)

Substituting in the first equation (Step 2) the concentration in mg.% was found to be 0.20 mg.%.

$$\frac{0.1352 - 0.0110}{0.3140} \times .5 = 0.20 \text{ mg.\%}$$

To calculate Total Ketosteroid Content, the concentration in mg.% was divided by 100 and multiplied by the total volume of the 24 hour specimen in mls. and found to be 7.11 mg. per Total Volume.

4. The results of analysis are reported in the following manner:

Total Volume	cc
17 Ketosteroids	mg.%
Total 17 Ketosteroids	mg./T.V.

Notes:

In order to constantly produce accurate results with this procedure, instructions should be very carefully followed. Slight errors can often produce abnormally high or low results.

The greatest errors in hydrolysis and extraction may be due to:

1. Too vigorous boiling during hydrolysis, or boiling longer than the prescribed 10 minutes. THIS IS ONE OF THE BIGGEST SOURCES OF ERROR.

2. Failure to completely remove phenolic and acidic impurities in the ether extract with 2N NaOH. The aqueous phase should be completely colorless during the last washing.

3. Failure to wash the last traces of NaOH from the ether extract with distilled water.

4. Failure to take up the residue from the ether extract in ethanol completely.

The most sensitive part of the procedure is in color development, and is the section in which most of the errors are made. Major sources of error include:

1. Inexact preparation of reagents; especially with 8N KOH. The normality of the KOH should be as nearly 8.000N as is possible, and the titer should be checked frequently - at least every 30 days.

2. Failure to allow all reagents to reach room temperature before beginning color development.

3. Improperly cleaned cuvettes. Cuvettes should be washed thoroughly, rinsed at least 3 times with tap water and finally, 3 times with distilled water. They should be thoroughly dry and free from dust and lint before using.

4. Inexact color development time. Color development should be exactly 20 minutes from the time 8N KOH is added to the cuvettes until diluting alcohol is finally added before reading.

5. Standard "S" Reading: All results depend greatly for their accuracy upon the reading of the standard. The standard MUST read from 45.00% to 51.00% transmission; 48.50% optimum. Whenever the Standard is not within this range, all cuvette solutions should be discarded and the whole color development procedure repeated, using clean cuvettes.

Interpretation:

17-Ketosteroids in varying quantities are normally excreted in the urine of all individuals, the amount depending upon age and sex. Normal 24 hour values include:

Adult Male	10 to 22 mg.
Adult Female	6 to 18 mg.
Children (under 5)	1 to 3 mg.
Children (5 to 12 yrs.)	2 to 6 mg.

This test serves as a valuable index of testicular and adrenocortical activity, and

certain diseases cause an abnormally high or low output. Disorders of the testes, adrenal cortex, and anterior pituitary can profoundly affect 17-ketosteroid excretion, rising as high as 800 mg. per diem in rare cases of masculinizing tumors of the testes, or dropping as low as 0.2 mg. per diem in Addison's disease or Panhypopituitarism.

1. Patient to take up the reaction from the first extract is obtained completely.

The most sensitive part of the procedure is in color development, and in the reaction in which most of the error is made. Major sources of error include:

1. Inexact measurement of reagents, especially with 5N KOH. The normality of the KOH should be as nearly 5.0N as is possible, and the flask should be checked for capacity - at least every 50 days.

2. Failure to allow all reagents to reach room temperature before beginning color development.

3. Inadequately cleaned reaction vessels. Vessels should be washed thoroughly, rinsed at least 3 times with tap water and finally, 2 times with distilled water. They should be thoroughly dry and free from dust and lint before using.

4. Inexact color development time. Color development should be exactly 30 minutes from the time 5N KOH is added to the reaction until dilution is finally added before reading.

5. Standard "S" Handling: All reagents should be kept for their full shelf life upon the receipt of the standard. The standard MUST read 48.50% to 51.50% transmittance at 48.50% solution. Whenever the standard is run within this range, all current solutions should be discarded and the whole color development process repeated, using these reagents.

Interpretation:

17-Ketosteroid results in varying quantities are normally reported in the units of mg. per diem, the amount depending upon age and sex. Normal 24 hour values include:

Adult Male	10 to 25 mg.
Adult Female	5 to 15 mg.
Children Under 5	1 to 5 mg.
Children (5 to 15 yrs.)	2 to 6 mg.

This test serves as a valuable index of testicular and adrenal cortical activity, and

LIPASE Of Pancreatitis

References:

1. Cherry, I. S., and Crandall, L. A., Am. J. Physiol. 100, 266 (1932)
2. Henry, R. J., Sobel, C., and Berkman, S., Clin. Chem. 3, 77 (1957)
3. Gomori, G., Am. J. Clin. Path. 27, 170 (1957)
4. Bunch, L. D., and Emerson, R. L., Clin. Chem. 2, 75 (1956)
5. Archibald, R. M., J. Biol. Chem. 165, 443 (1946)

Principle:

Serum is incubated with an olive-oil emulsion substrate. Lipase activity results in splitting of the glyceryl-fatty acid ester bond with the liberation of free fatty acids. The amount of action is determined by titrating the liberated fatty acids with standard alkali using thymolphthalein as the indicator. A glass electrode pH meter may also be used to detect the end-point.

Reagents:

1. Olive-oil emulsion

Gum acacia 12.5 g.
Olive oil, 50 ml.
Distilled water 100 ml.
Sodium benzoate 0.2 g.

Olive oil may be purified as follows:

Wash 100 ml. of olive oil with thorough shaking in a separatory funnel, once with 100 ml. of 0.5% NaHCO_3 and then twice with water.

Dissolve the gum acacia and the sodium benzoate in the water. Add the olive oil and emulsify by passing the mixture through a hand homogenizer until a smooth emulsion results, usually 3 to 5 times. Store in the refrigerator. Shake well before using. Preferably pass the emulsion through the homogenizer just prior to use or prepare fresh each day.

As an alternate preparation method, emulsify at high speed for 15 minutes in a Waring blender.

2. Phosphate buffer 0.67 M pH 7.5

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 15.7 g.
 KH_2PO_4 anhyd. 1.2 g.

Dissolve the salts in distilled water and dilute up to about 1 liter. Add one ml. toluene and keep in refrigerator.

3. Alcohol-ether mixture.

Add 9 parts (by volume) of 95% ethyl alcohol to 1 part diethyl-ether.

4. Thymolphthalein indicator.

0.4 g. of the indicator are dissolved in 50 ml. 95% ethyl alcohol and then 50 ml. of distilled water are added.

5. 0.05 N NaOH. The precise normality must be known.

Dilute 5 ml. of 2.5 N NaOH up to 250 ml. and standardize against standard acid using thymolphthalein as the indicator.

Procedure:

1. Prepare substrate-buffer mixture by mixing 5 volumes of phosphate buffer with one volume of olive oil emulsion by stirring. The pH of the buffered substrate should be close to 7.5.
2. Measure 12 ml. portions of the substrate-buffer into test tubes (or flasks) using 2 tubes for each determination. Warm the tubes of substrate buffer mixture to 37° in a water bath.
3. Add 1.0 ml. serum to one of the tubes and mix thoroughly by gentle inversion. The second tube will serve as the blank. Incubate both tubes for 4 or for 16-24 hours at 37°C.
4. At the end of the incubation period, add 1.0 ml. of serum to the blank.
5. Immediately pour the contents of the tubes into a 100 ml. beaker and transfer completely by rinsing out the tube with 50 ml. of the 9:1 alcohol-ether mixture. Mix well with a glass stirring rod.
6. Titrate the mixtures with 0.05 N NaOH to a "distinct" blue using 4-6 drops of thymolphthalein indicator (or titrate to pH 10.65 using a glass electrode pH meter.)

Calculation:

The final result is expressed in terms of the volume of 0.05 N NaOH required to titrate the fatty acid liberated by 1 ml. of serum in 4 or in 16-24 hours.

Example: A serum sample gave these results for the blank and test sample:

Test sample	8.20 ml. 0.06 N NaOH
Blank "	3.30 ml. 0.06 N NaOH
	4.90 ml. difference

Since 0.06 N NaOH was used

$$4.90 \times (0.06/0.05) = 5.88 \text{ units/ml. serum}$$

Interpretation:

The determination of "lipase" is most often requested as an aid in the diagnosis of acute pancreatitis. The lipolytic enzyme found in serum of patients with acute pancreatitis apparently is inactive when tested with substrates of low molecular weight such as tributyrin or ethyl butyrate, but active when tested with high molecular weight substrates such as olive oil or triolein emulsions. (See Esterase, p. 164.)

Normally there are present in serum lipolytic enzymes (esterases) active when tested with low molecular weight substrates but little or none of the enzyme active when tested with high molecular weight substrates (lipases).

The values found for "lipase" in cases of certain disease states are listed below:

<u>Condition</u>	<u>4 hour lipase</u>	<u>16-24 hr. lipase</u>
Normal	0.31 (0.06 - 0.89)	(0.2 - 1.5)
Acute pancreatitis	2 - 10	similarly elevated

Note: 1. The incubation may be carried out in Erlenmeyer flasks to avoid subsequent transfers.

2. The addition of alcohol-ether may be eliminated, if preferred, if good stirring and a slow approach to the end point is made.

MAGNESIUM (Titan Yellow) Serum

References:

- Neill, D. W. and R. A. Neely; The estimation of magnesium in serum using titan yellow. *J. Clin. Path.*, 9, 162 (1956).
Heagy, F. C.; Use of polyvinyl alcohol in the colorimetric determination of magnesium in plasma or serum by means of titan yellow. *Can. J. Research*, 26E, 295 (1948).

Principle:

The blood, serum, or plasma proteins are precipitated by means of tungstic acid. An aqueous solution of titan yellow is added to the water-clear filtrate, and sodium hydroxide is added to develop the red magnesium hydroxide-titan yellow complex. Gum ghatti is used to stabilize the color lake.

Reagents:

Gum ghatti 0.1%. Powdered gum ghatti, 0.1 g., is suspended in a muslin bag in 100 ml. of distilled water for 24 hours. (Polyvinyl alcohol 0.1% can be used in place of gum ghatti.)

Titan yellow 0.05%. The dye powder, 0.1 g., is dissolved in 200 ml. distilled water.

Stock Standard Magnesium Chloride. 8.458 g. of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, is dissolved in distilled water and made up to one liter. This solution contains 1,000 micrograms magnesium per ml.

Working Standard Magnesium Chloride. One ml. stock standard solution is diluted to 200 ml. with distilled water to give a concentration of 5 micrograms of magnesium per ml. Volumes of 1, 2, 3, 4, and 5 ml. made up to 5 ml. with distilled water in each case and representing 5, 10, 15, 20 and 25 micrograms of magnesium are used in setting up the standard curve.

Calcium Chloride. 16.13 mg. of $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ is dissolved in distilled water and made up to 100 ml., to give a final concentration of 0.05 mg. Ca per ml.

Sulfuric Acid 0.67 N (reagent grade).

Sodium hydroxide 4 N (reagent grade).

Sodium tungstate ($\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$) 10%.

Procedure:

1. One ml. of serum is diluted with 5 ml. of distilled water.
2. Add 2 ml. of 10% sodium tungstate and 2 ml. of 0.67 N H_2SO_4 .
3. Mix and then centrifuge for 5 minutes at 2500 rpm.
4. To 5 ml. of the protein-free supernatant is added 1 ml. distilled water, 1 ml. 0.1% gum ghatti, 1 ml. 0.05% titan yellow solution and 2 ml. 4 N NaOH.
5. A reagent blank using 1 ml. CaCl_2 solution containing 0.05 mg. Calcium in place of serum is treated similarly to the test.
6. Compare photometrically at 540 mu. Set the photometer to read 100% transmittance against water.

Standard Curve:

Readings of optical density are converted to magnesium concentrations by reference to a standard curve.

Prepare the standard curve by carrying out the above color reactions on 5 ml. samples of solutions containing 5, 10, 15, 20, and 25 micrograms of magnesium replacing the 1 ml. of distilled water added to the protein-free supernatant in the test with 1 ml. CaCl_2 solution containing 0.05 mg. calcium. The standard blank is 5 ml. of distilled water.

Calculations:

$$\text{Magnesium} = \frac{\text{O.D.}_T - \text{O.D.}_B}{\text{O.D.}_S - \text{O.D.}_B} \times C_S \times \frac{100}{0.5}$$

where: C_S = amount of Mg in standard in mg.

O.D._T = optical density of test solution

O.D._S = optical density of standard

O.D._B = optical density of reagent blank

Example.

$$\text{O.D.}_T = 0.600$$

$$\text{O.D.}_S = 0.575$$

$$\text{O.D.}_B = 0.515$$

$$C_S = 0.01$$

$$\text{Then: } \frac{0.600 - 0.515}{0.575 - 0.515} \times 0.01 \times \frac{100}{0.5} = 2.83 \text{ mg. Mg/100 ml.}$$

The value can also be obtained from the standard curve.

Interpretation:

Normally about 1 to 3 mg. of Mg are found in 100 ml. of serum and about 1.6 mg. per 100 ml. of blood.

Decreased Mg levels have been observed in severe renal disease, toxemia of pregnancy and chronic alcoholism. Increased Mg levels have been observed in Mg poisoning from the ingestion of epsom salt in severe renal disease.

MAGNESIUM

Serum

References:

1. Briggs, A. P., J. Biol. Chem. 52, 349-355 (1922).
2. Dennis, W., J. Biol. Chem. 52, 411 (1922).
3. Fiske, C. H., J. Biol. Chem. 66, 375 (1925).

Principle:

Magnesium is determined in the serum following removal of calcium. The magnesium is precipitated as magnesium ammonium phosphate ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$). This compound is treated with ammonium molybdate to form phosphomolybdic acid, which is then reduced to molybdenum blue by semidine or amino-naphthol-sulfonic acid. The molybdenum blue (uncertain composition) is then determined photometrically.

Reagents:

1. 2% (w/v) potassium dihydrogen phosphate. Dissolve 2 g. KH_2PO_4 (reagent grade) in 100 ml. water. Filter this solution each time just before use.
2. Ammonium hydroxide. Concentrated reagent grade (sp.gr. 0.9).
3. Alcoholic wash solution. Add 200 ml. of 95% (v/v) ethyl alcohol and 50 ml. concentrated ammonium hydroxide to a 1000 ml. volumetric flask. Fill to the mark with water and mix thoroughly. Keep in refrigerator.
4. Ammonium molybdate reagent. 0.0083 N. Place 2.566 g. of reagent grade ammonium molybdate in a 250 ml. volumetric flask. Add slowly about 175 ml. of distilled water and 35 ml. concentrated H_2SO_4 (sp. gr. 1.84) while swirling the flask. Then add water to the level of the neck of the flask and allow to cool. Now add water to the mark and mix.
5. N-phenyl-p-phenylenediamine monohydrochloride (semidine-Eastman Kodak Co. #2043). Place 50 mg. of the salt in a 100 ml. volumetric flask. Wet the salt with a few drops of ethanol and fill the flask to the mark with 1% aqueous sodium bisulfite solution. Shake the flask vigorously while filling. Filter the resultant cloudy, purplish solution, and store the colorless, clear filtrate in a brown bottle in the refrigerator. Discard this reagent when the blank becomes excessively colored.
6. Amino-naphthol-sulfonic acid (may be used instead of semidine). Dissolve 30 g. of sodium bisulfite (NaHSO_3) and 1 g. of sodium sulfite ($\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$) in 200 ml. water. Add 0.5 g. of 1-amino-2-naphthol-4-sulfonic acid and mix thoroughly. Place in a dark glass bottle. This colorless solution develops a yellowish discoloration after about two weeks. Thus, it must be discarded at that stage.
7. Phosphorus stock standard. Place 438.1 mg. of potassium dihydrogen phosphate into a 100 ml. volumetric flask. Add water to the mark. Dilute 20 ml. of the stock standard to 1000 ml. with distilled water for use as a working standard. Each ml. of the working standard contains 0.02 mg. of phosphorus.

Procedure:

1. Follow steps 1-6 of the calcium procedure on page 119. Transfer the supernate obtained in Step 6 to a 12 ml. heavy duty centrifuge tube.
2. Add 1 ml. of 2% KH_2PO_4 and 1 ml. of concentrated ammonium hydroxide to this centrifuge tube.
3. Mix thoroughly and help initiate precipitation by rubbing the inside of the tube with a glass rod.
4. Rinse glass rod with several drops of water before removing.
5. Stopper the tube and place in refrigerator for 2 hours.
6. Centrifuge at 1500 rpm for 30 minutes.
7. Decant supernate carefully and discard.
8. Add 5 ml. of chilled alcoholic wash fluid. Tap tube until the precipitate is re-suspended.
9. Centrifuge at 1500 rpm for 30 minutes and again discard the supernate.
10. Add 5 ml. water and tap the tube to resuspend the precipitate.
11. Prepare 2 standards in similar tubes. Place 2 and 4 ml. of phosphate working standard in these tubes respectively. Dilute to 5 ml. (by adding 3 and 1 ml. H_2O respectively).
12. To each tube add 0.5 ml. molybdate reagent.
13. Prepare the blank by adding 5 ml. water and 0.5 ml. molybdate in a similar tube.
14. To each tube add either 5 ml. semidine reagent or 0.4 ml. amino-naphthol-sulfonic acid reagent. Mix each tube immediately after addition of this reagent.
15. Let stand five minutes. Compare photometrically in a photometer at 660 mu.

Calculations:

1. In a molecule of MgNH_4PO_4 , 31 mg. of phosphorus is equivalent to 24 mg. of magnesium.
2. The working phosphate standard contains 0.02 mg. P/ml.

$$\frac{0.04 \text{ mg. P}}{2 \text{ ml.}} \times \frac{24 \text{ mg. Mg.}}{31 \text{ mg. P}} \times \frac{1 \text{ meq. Mg.}}{12 \text{ mg. Mg.}} \times 1000 = 1.29 \text{ meq. Mg./liter}$$

a. Thus the 2 ml. standard represents 1.29 meq. Mg./liter.

b. The 4 ml. standard represents 2.58 meq. Mg./liter.

3. A calibration curve may be prepared by plotting optical density versus Mg in meq./liter on equal axis graph paper or the calculation may be made using the nearest standard.

$$(\text{D}_s/\text{D}_s) \times C_s \times \frac{1000}{2} = \text{Mg (meq./liter)}$$

$$\text{For 2 ml. standard} = (\text{D}_u/\text{D}_s) \times 0.00258 \times \frac{1000}{2} \text{ meq./liter}$$

$$= (\text{D}_u/\text{D}_s) \times 1.29 \text{ meq./liter}$$

$$\text{For 4 ml. standard} = (\text{D}_u/\text{D}_s) \times 2.58 \text{ meq./liter}$$

NITROGEN (TOTAL)
Macro-Kjeldahl Method

Reference:

Hiller, A., Plazin, J., and Van Slyke, D.D.: J. Biol. Chem. 176, 1401 (1948)

Principle:

By digestion with concentrated H_2SO_4 and a catalyst such as copper sulphate, all of the nitrogen of the sample is converted to $(NH_4)_2SO_4$. The ammonia is then liberated by making the solution strongly alkaline with NaOH. Distillation of the NH_3 into boric acid solution is then carried out. The NH_3 in the boric acid is then titrated with standard acid.

Reagents:

1. Concentrated H_2SO_4
2. 10% $CuSO_4 \cdot 5H_2O$
3. 40% NaOH. Dissolve 40 g. of NaOH in water. Cool. Dilute to about 100 ml.
4. 0.1 N HCl, standardized
5. Boric Acid, 2%
6. Mixed indicator, five parts of 0.1% bromocresol green in alcohol plus one part of 0.1% methyl red in alcohol
7. Zinc metal, mossy

Procedure:

1. Into a 300 ml. Kjeldahl flask pipet exactly 5 ml. of urine or 1.0 ml. of serum (or other volumes of other materials; they should contain between 1.0 and 5.0 mEq. of nitrogen). Add accurately with a cylinder 10 ml. of conc. H_2SO_4 . Add 2 ml. of 10% $CuSO_4 \cdot 5H_2O$ solution.
2. Digest on a digestion rack until the solution is clear and faintly blue-green in color.
3. Cool. Add 150 ml. of H_2O . Mix. Cool.
4. Pour in, to run under the acid layer, 45 ml. of 40% NaOH solution. (DANGER! Do not spill!)

The acid layer above the NaOH prevents loss of ammonia. Add a small piece of mossy zinc to promote smooth boiling.

Without shaking connect the flask through a spray trap to a condenser.

5. Into a 250 ml. Erlenmeyer flask place 50 ml. (measured with a cylinder) of 2% boric acid, and 0.5 ml. of mixed indicator solution. place the flask so that the glass delivery tube at the end of the condenser dips just below the surface of the acid. Start water flowing around the condenser.

6. Now shake the Kjeldahl flask to mix up the acid and alkaline solutions. A deep blue color should appear. Heat the Kjeldahl flask and distill 100 ml. of liquid into the Erlenmeyer flask.

7. When 100 ml. have distilled over, lower the Erlenmeyer flask until the delivery tube is out of the solution and distill a few ml. more to rinse out the inside of the tube.

Rinse outside of delivery tube with distilled water using a wash bottle.

9. Titrate the ammonia with 0.1 N HCl to the loss of blue-green color and the appearance of a "gray" or neutral color. See discussion below. Determine the "blank" correction as directed below and subtract it from the volume of acid used.

Calculations:

Take N as the normality of the standard acid, of which a total of A ml. was used, B as the blank correction, and V as the volume of 24-hour urine. Since a 1 molar solution of NH_3 contains 14 g. of N per liter,

$$(A - B) \times N \times 0.014 \times (V/5) = \text{g. nitrogen per 24 hours}$$

Discussion:

The color change of the indicator is indicated by the following data: Blue purple - pH 4.5; Blue-green - pH 4.9; pink - pH 4.3. The end point being between the pink and the blue - where the solution appears gray.

To determine the blank, introduce into a thoroughly clean 250 ml. Erlenmeyer flask 50 ml. of 2% boric acid, and 0.5 ml. of mixed indicator solution. Add water to volume of the unknowns and titrate as above. This represents a titration (or indicator) blank. For a complete reagent blank a complete digestion and distillation should be carried out without adding a sample.

NON-PROTEIN NITROGEN IN BLOOD

Micro-Kjeldahl Method

Reference:

Folin, O., and Wu, H.: J. Biol. Chem. **38**, 81 (1919)

Hoffman, W.S.: J. Lab. and Clin. Med., **25**, 856 (1940)

Principle:

A protein-free filtrate is first prepared by the tungstic acid procedure. Then all organic compounds present in protein-free filtrate are destroyed by oxidation with a mixture of H_2SO_4 and H_3PO_4 . All nitrogen present in these compounds is converted to NH_3 . The solution is diluted and added to alkaline Nessler's reagent, developing color in proportion to the amount of ammonium ion. Photometric comparison is made with a solution containing a known amount of nitrogen in the form of ammonium ion.

Reagents:

Use ammonia-free distilled water for all solutions and dilutions.

1. Ammonium sulfate, stock solution. Dry some pure ammonium sulfate in a vacuum desiccator over a drying agent for 24 hours. Weigh out 0.9434 g., dissolve in water, add 1 drop of conc. sulfuric acid, dilute to exactly 1 liter, and mix. 1 ml. contains 0.2 mg. of nitrogen.

2. Ammonium sulfate solution, dilute standard. Dilute exactly 15 ml. of the stock solution up to 100 ml. in a volumetric flask and mix. 5 ml. contains 0.15 mg. of nitrogen.

3. Alkaline Nessler's reagent for acid digestion mixtures. Dilute 135 ml. of stock mercuric potassium iodide solution (see Urea Nitrogen, p. 265) plus 800 ml. of 2.5 N NaOH with water up to 1 liter and mix. This solution should be made several days ahead of use. Any sediment that forms should be allowed to settle, and only clear supernatant liquid should be used. The amount of alkali given here is adapted for the use of 15 ml. of the reagent in a total volume of 50 ml. of Nesslerized solution, when 20 milliequivalents of an acid are to be neutralized in the digestion mixture.

4. Acid digestion mixture for non-protein nitrogen. Dissolve 0.6 g. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 100 ml. of water, add 75 ml. of conc. H_2SO_4 and 25 ml. of 85% H_3PO_4 and mix.

5. 2% Gum ghatti solution. Tie 2 g. of gum ghatti tears in a bit of washed gauze and hang for 24 hours near the top of 100 ml. of water in a cylinder. Use a few drops of toluene as a preservative. Discard the undissolved portion, mix, and filter through cotton. Keep in the refrigerator. Use 1 drop (about 0.05 ml.) for each 10 ml. of final volume of Nesslerized solution. This acts as a protective colloid.

Procedure:

1. Into a 25 x 200 mm. pyrex test tube graduated at 35 ml. place 5 ml. of tungstic acid protein-free blood filtrate. Add 1 ml. of acid digestion mixture and a glass bead to prevent bumping. Using a microburner, boil off the water. Lower the flame as low as possible, and continue to heat until acid fumes (dense white) fill the tube. Then cover with a watch glass or a small funnel. See that the heat from the small flame does not strike the tube above the surface of the liquid.

2. The liquid turns dark and then grows lighter. As soon as all brown or yellow color has disappeared, and the color is a faint blue-green, cease heating. The heating period should be as short as possible.

3. Allow to cool until the tube can be handled.

4. Add about 5 ml. of water and make sure all the syrupy liquid goes into solution. Dilute to about 30 ml. and add 5 drops of 2% gum ghatti solution. Cool in a water bath with the standard to about 20°C. Dilute to exactly 35 ml. In a clean, dry 100 ml. beaker place exactly 15 ml. of Nessler's reagent (for acid digestion mixtures). Rotate the latter and pour in rapidly the 35 ml. of unknown solution. Pour back and forth several times to mix.

5. Prepare a standard solution. In a test tube graduated at 35 ml., place 5 ml. of standard ammonium sulfate solution containing 0.15 mg. of nitrogen. Add 1 ml. of above (no heating required). Nesslerize the standard, the unknown, and the blank simultaneously and read in the photometer (515 mμ) after 10 to 20 minutes. The blank containing digestion mixture and gum ghatti, is treated in the same way as the standard.

Calculations:

5 ml. of tungstic acid filtrate corresponds to 0.5 ml. of blood. Then the non-protein nitrogen (NPN) found is:

$$(D_u/D_s) \times 0.15 \times (100/0.5) = (D_u/D_s) \times 30 \text{ (mg. N.P.N./100 ml. blood)}$$

Comments:

If a concentrated solution of phosphoric acid is heated too long or at too high a temperature, it attacks glass. The resulting silicious material may cause turbidity at a later step. Such turbidity most often appears when there has been carelessness or lack of skill in the heating.

The process of Nesslerization is used frequently in biochemical work. After mixing the color increases gradually. Color is due to a colloidal solution of $\text{NH}_2\text{Hg}_2\text{I}_3$ (formula in doubt). See Nichols and Willits: J. Am. Chem. Soc. 56, 769-774 (1934). This complex salt is very insoluble and can be used for gravimetric determination of ammonia in large amounts. However, it can be made, by proper selection of conditions, to form a clear colloidal solution well adapted to photometric determination. Once the Nesslerized solution becomes turbid the analysis is ruined. The necessary conditions for securing clear solutions are:

1. The concentration of ammonia (NH_4^+) should not be too high (less than 1 mg. per ml. of final solution).

2. Proper alkalinity. Free OH^- ions should be present at a concentration of 20 MEq per 100 ml. of final solution.

3. Temperature of the solution should be low--20°C. or lower.

4. Mixing of the alkaline Nessler reagent with the ammonia solution must be instantaneous. Otherwise for an appreciable length of time, an unsuitable concentration of OH^- ions will exist, and the precipitation of the colloid may begin.

5. The presence of a protective colloid (gum ghatti) greatly facilitates the maintenance of the colloidal state.

6. The concentration of neutral salts should be low.

7. See also p. 266.

4. Sodium hydroxide 0.5 N. Standardize and adjust to between 0.49 and 0.51 N.
5. Sodium bicarbonate 0.5 M.
Dissolve 4.2 g. NaHCO_3 in distilled water and dilute up to 100 ml.
6. 4-amino-antipyrine 0.6% (w/v).
Dissolve 0.6 g. of 4-amino-antipyrine in distilled water and make up to 100 ml.
7. Potassium ferricyanide 2.4%.
Dissolve 2.4 g. $\text{K}_3\text{Fe}(\text{CN})_6$ in distilled water and dilute up to 100 ml.
8. Stock phenol standard
Dissolve 1.2 g. pure crystalline phenol and dilute up to 1 liter using about 0.1 N HCl as the solvent (8.5 ml. concentrated HCl diluted up to 1 liter). Standardize as outlined below.
9. Dilute phenol standards
 - (a) Weak working standard.
Dilute 1.00 ml. stock phenol standard up to 100 ml. with distilled water. Add 2-3 drops of toluene. (1 ml. = 0.01 mg.)
 - (b) Strong working standard.
Dilute 3.00 ml. stock phenol standard up to 100 ml. with distilled water. Add 2-3 drops of toluene. (1 ml. = 0.03 mg.)
 Store under refrigeration. Good for one month.
10. Tartrate buffer 0.2 M pH 4.9
Dissolve 3.00 g. L(+) tartaric acid-analytical grade (dextro-rotatory tartaric acid) in 50 ml. distilled water. Add about 35 ml. 1 N NaOH, and adjust the pH to 4.9. Dilute to 100 ml. Add two drops of toluene and store under refrigeration.

Procedure:

Into a large test tube whose contents can be easily mixed by shaking carry out the additions and operations listed below in the tables.

<u>A. Control and Test</u>	<u>Control (ml.)</u>	<u>Test (ml.)</u>
Carbonate Buffer	1.0	1.0
Substrate	1.0	1.0
Mix and warm 3 minutes at 37°C. in a water bath.		
Plasma		0.1
Mix and warm 15 minutes at 37°C. in a water bath.		
NaOH 0.5 N	0.8 mix	0.8 mix
Plasma	0.1 mix	---
NaHCO_3 0.5 M	1.2 mix	1.2 mix
A.A.P. 0.6%	1.0 mix	1.0 mix
$\text{K}_3\text{Fe}(\text{CN})_6$ 2.4%	1.0 mix	1.0 mix

B. Blank and Standards

	<u>Blank</u>	<u>Standard</u> (0.01)	<u>Standard</u> (0.03)
Carbonate buffer	1.1	1.1	1.1
Phenol standard			
0.01	---	1.0	---
0.03	---	---	1.0
Water	1.0	---	---
NaOH 0.5 N	0.8 mix	0.8 mix	0.8 mix
NaHCO ₃ 0.5 M	1.2 mix	1.2 mix	1.2 mix
A. A. P. 0.6%	1.0 mix	1.0 mix	1.0 mix
K ₃ Fe(CN) ₆ 2.4%	1.0 mix	1.0 mix	1.0 mix

Acid Phosphatase

A. Control and Test

	<u>Control</u>	<u>Test</u>
Citrate buffer	1.0	1.0
Substrate	1.0	1.0
	Mix and warm - 3 minutes 37°C.	
Plasma	---	0.1 ml.
	Mix and warm - 1 hour 37°C.	
NaOH 0.5 N	1.0 mix	1.0 mix
Plasma	0.1 ml.	---
NaHCO ₃ 0.5 M	1.0 mix	1.0 mix
A. A. P. 0.6%	1.0 mix	1.0 mix
K ₃ Fe(CN) ₆ 2.4%	1.0 mix	1.0 mix

B. Blank and Standards

	<u>Blank</u>	<u>Standard₁</u>	<u>Standard₂</u>
Citrate buffer	1.1	1.1	1.1
Phenol standard			
0.01	---	1.0	---
0.03	---	---	1.0
Water	1.0	---	---
NaOH 0.5 N	1.0 mix	1.0 mix	1.0 mix
NaHCO ₃ 0.5 M	1.0 mix	1.0 mix	1.0 mix
A. A. P. 0.6%	1.0 mix	1.0 mix	1.0 mix
K ₃ Fe(CN) ₆ 2.4%	1.0 mix	1.0 mix	1.0 mix

Allow the color to develop for 15 minutes and read against a distilled water optical blank at 510 mu. If the transmittance of any of the unknowns is between 5 and 10%, the analysis may be saved by diluting control and test solutions, and blank and standard solutions, with an equal volume of water. Mix and compare photometrically, as before. The color may fade rapidly after 60 to 120 minutes due to (a) phenol or (b) reducing substances.

Calculation:

A King-Armstrong unit of alkaline phosphatase is defined as the amount of enzyme which will liberate one mg. of phenol from a 0.005 M solution of disodium phenyl phosphate in 15 minutes at 37°C. in bicarbonate buffer at pH 10.

A King-Armstrong unit of acid phosphatase is defined as the amount of enzyme which will liberate one mg. of phenol from a 0.005 M solution of disodium phenyl phosphate in one hour at 37°C. in citrate buffer at pH 4.9.

$$\text{Phosphatase units} = \frac{D_t - D_c}{D_s - D_b} \times 0.01 \text{ (or } 0.03) \times \frac{100}{0.1}$$

$$= \frac{D_t - D_c}{D_s - D_b} \times 10 \text{ (or } 30)$$

where D refers to optical densities of

t = test; c = control; s = standard and b = blank

Prostatic Acid Phosphatase (tartrate sensitive fraction)

Proceed as under acid phosphatase but use a mixture of 8 parts citrate buffer pH 4.9 and 2 parts 0.2 M tartrate buffer pH 4.9. It is not necessary to use tartrate buffer for the blank, standards or the control tube since the color production is not affected by tartrate.

Interpretation:

Alkaline Phosphatase.

Normal values - adults 4-10 King-Armstrong units

-children 10 - 20 King-Armstrong units

Elevated values are seen in disorders of bone metabolism and in hepatic and biliary tract disease; for example, rickets, hyperparathyroidism, hepatocellular damage, and biliary obstruction.

Acid Phosphatase

Normal values 0 - 4 King-Armstrong units

Increased values are seen in prostatic carcinoma with metastases. Levels which are inhibited to an extent of more than one King-Armstrong unit by tartrate are said to be indicative of prostatic malignancy.

Standardization of Phenol

Reagents:

1. Phenol stock standard - about 1 mg. per ml.

2. Sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3$) 0.1 N.

Prepare as outlined under Secondary Standards, p. 29.

3. Iodine-potassium iodide solution 0.1 N. Dissolve 25 g. potassium iodide (KI) in 25 ml. of distilled water, add 12.7 g. resublimed (analytical grade) I_2 , dissolve and dilute up to 1 liter.

4. Potassium bi-iodate $\text{KH}(\text{IO}_3)_2$ 0.1 N (or Potassium iodate KIO_3 0.1 N.) Prepare as outlined under Primary Standards, p. 23.

Procedure: See p. 29, Iodimetry.

A. Standardization of 0.1 N sodium thiosulfate

1. Into a 250 ml. Erlenmeyer flask pipet 5 ml. of 6 N hydrochloric acid, add 2 g. potassium iodide and 3-5 ml. of water.

2. Transfer 25.00 ml. of standard bi-iodate (or iodate) to the flask. Free iodine will now appear in the solution.

3. Titrate the liberated iodine with 0.1 N sodium thiosulfate and calculate the thiosulfate concentration. (See p. 18 Volumetric calculations.)

B. Standardization of Iodine-potassium iodide 0.1 N solution.

1. 25.00 ml. of $\text{I}_2 \cdot \text{KI}$ solution are transferred to a 250 ml. Erlenmeyer flask and titrated with the standardized sodium thiosulfate solution.

2. Calculate the iodine concentration as above (A3).

C. Standardization of stock phenol standard.

1. Transfer 25.00 ml. of stock phenol solution to a 250 ml. glass-stoppered Erlenmeyer flask. Add 50 ml. 0.1 N NaOH and heat to 65°C . (not higher). Add 25.00 ml. of the previously standardized iodine-potassium iodide solution and mix.

2. Stopper the flask and allow to stand at room temperature for 45 minutes.

3. Add 5 ml. of concentrated hydrochloric acid and titrate the excess iodine with the previously standardized sodium thiosulfate solution.

Calculation:

Mg. phenol per 25 ml. = $1.568 \times (\text{ml. } 0.1 \text{ N } \text{I}_2 - \text{ml. } 0.1 \text{ N thiosulfate.})$ Adjust the concentration of phenol to exactly 1.00 mg. per ml. and store under refrigeration.

Example:

A. Standardization of 0.1 N sodium thiosulfate 23.45 ml. of sodium thiosulfate were required to titrate 25.00 ml. 0.1 N $\text{KH}(\text{IO}_3)_2$

$$\frac{25.00}{23.45} \times 0.1 = 0.1065 \text{ N sodium thiosulfate}$$

Factor is 1.065

B. Standardization of $\text{I}_2 \cdot \text{KI}$ solution. 24.87 ml. of 0.1065 N sodium thiosulfate

were required to titrate 25.00 ml. of $I_2 \cdot KI$ solution.

$$\frac{24.87 \times 0.1065}{25.00} = 0.1060 \text{ N } I_2 \cdot KI \text{ solution}$$

Factor is 1.060

C. Standardization of stock phenol standard solution. 6.90 ml. of 0.1065 N sodium thiosulfate were required to titrate the iodine excess.

$$\text{Mg. phenol per 25 ml.} = 1.568 (25.00 \times 0.1060 - 6.90 \times 0.1065)$$

$$= 1.568 (26.50 - 7.35)$$

$$= 1.568 \times 19.15 = 30.00 \text{ mg. phenol}$$

D. Dilution Calculation,

$$\frac{30.00}{25.00} = 1.2 \text{ mg. phenol per ml.}$$

If 970 ml. of the solution remain:

$$970 \times 1.2 = X \times 1.0$$

$$X = \frac{970 \times 1.2}{1.0} = 1163 \text{ ml.}$$

$$1163 - 970 = 193 \text{ ml. } 0.1 \text{ N HCl to be added to give 1.00 mg. phenol per ml.}$$

PHOSPHATE, INORGANIC Serum

References:

1. Fiske, C.H. and Subbarow, Y., J. Biol.Chem. 66, 375 (1925)
2. Gomori, G., J. Lab. & Clin. Med. 27, 955 (1941-42)
3. Dryer, R.L., Tammes, A.R., and Routh, J.I., J. Biol. Chem. 222, 177 (1957)

Principle:

The inorganic phosphorus present in a trichloroacetic acid protein-free filtrate of serum in the form of the orthophosphate (salts of orthophosphoric acid, H_3PO_4) react with molybdate in acid solution, and a blue color is formed from the resultant "phosphomolybdate" after the addition of a reducing agent, such as "pictol" or "metol" which is p-methylamino-phenol (sulfate).

Reagents:

1. Trichloroacetic acid 5% w/v in water.)
Trichloroacetic acid 10% w/v in water.) **Keep refrigerated! Important!**
2. Sulfuric acid 10 N. Carefully pour 100 ml. of concentrated H_2SO_4 into 200 ml. of water, in a mixing cylinder (Pyrex), mix. After cooling dilute to 360 ml. (exactly) with water.
3. Molybdate II for serum (2.5% ammonium molybdate in 3.0 N H_2SO_4). In a 1 liter mixing cylinder dissolve 25 g. of the salt in 300 ml. of 10 N H_2SO_4 and dilute to a liter with water.
4. "Pictol" stock. Dissolve 3 g. $NaHSO_3$ in 100 ml. H_2O and add 1 g. "pictol" (Mallinckrodt). Shake. Store in brown bottle. Refrigerate. It will keep indefinitely.
5. Dilute "pictol." To 10 ml. "pictol" stock add 90 ml. H_2O . Mix well. Prepare on day of use.
6. Stock standard phosphate, KH_2PO_4 is well dried. 0.3514 g. transferred to a 1 liter flask + 10 ml. 10 N H_2SO_4 , dilute to mark (5 ml. = 0.4 mg. P).
7. Dilute standards: Prepare 2 standard solutions weekly as follows from stock phosphate.
 - a. Strong working standard:
Transfer 5 ml. stock std. phosphate to 100 ml. vol. flask, dilute to mark with 5% trichloroacetic acid (5 ml. = 0.02 mgm.).
 - b. Weak working standard:
Same except use 2 ml. (5 ml. = 0.008 mgm.).

Procedure:

1. To a glass-stoppered test tube or a glass-stoppered bottle, add exactly 9.0 ml. of 10% trichloroacetic acid.
2. Add exactly 1.0 ml. of serum, mix well. Allow to stand 5-10 minutes and centrifuge until clear or filter through a #40 Whatman filter paper.
3. To a cuvet or colorimeter tube add exactly 5.0 ml. of filtrate.

4. Prepare a blank by pipetting 5.0 ml. of 5% trichloroacetic into a cuvet.
5. Prepare two standards in a similar manner.
 - a. 5 ml. = 0.02 mg.
 - b. 5 ml. = 0.008 mg.
6. Add 1.0 ml. molybdate II to each cuvet and mix.
7. Add 4.0 ml. dilute pictol to each cuvet, mixing immediately after the addition to each cuvet in succession.
8. Read in a photometer after 10 minutes and before 60 minutes at 630 mu (or using a red filter) against the reagent blank set at 100.

Calculation:

$$\text{Mg. P/100 ml. serum} = (D_u/D_s) \times C_s \times \frac{100}{0.5}$$

$$\text{for strong standard: } (D_u/D_s) \times 0.02 \times \frac{100}{0.5} = (D_u/D_s) \times 4 = \text{mg. per cent}$$

$$\text{for weak standard: } (D_u/D_s) \times 0.008 \times \frac{100}{0.5} = (D_u/D_s) \times 1.6 = \text{mg. per cent}$$

Urine:

1. Dilute exactly 1.0 ml. of urine to 50 ml. with 5% trichloroacetic acid in a 50 ml. volumetric flask, mix well. Filter if not perfectly clear through Whatman #40 filter paper.
2. To a cuvet add 5.0 ml. diluted urine.
3. Add 1.0 ml. molybdate II.
4. Add 5.0 ml. dilute pictol and mix immediately.
5. Compare in a photometer with blank and standard prepared as above for serum.

Phosphatase, Alkaline and Acid:

Reference:

Shinowara, G. Y., Jones, L. M., and Reinhard; The Estimation of Serum Inorganic Phosphorus and Acid and Alkaline Phosphatase Activities. *J. Biol. Chem.* **142**, 921 (1942).

Principle:

The alkaline and acid phosphatase activity of serum is estimated by allowing serum to incubate at 37°C. with an organic phosphate ester, (B-glycerophosphate) at an appropriate pH, pH 8.7 for alkaline and 5.0 for acid phosphatase. The liberated inorganic phosphorus is then determined

The Bodansky phosphatase unit is defined as that amount of phosphatase which will liberate one mg. of inorganic P from the substrate in one hour.

Apparatus:

1. As for phosphate.
2. Glass-stoppered bottles.
3. Incubator set at 37°C.

Reagents:

1. As for inorganic phosphorus. (see above).
2. Buffered substrate-stock solution. A mixture of:
Sodium B-glycerophosphate 2.50 g. and
Sodium barbiturate (sodium barbitol USP) 2.12 g. is diluted up to 250 ml.
Mix. Preserve stock substrate in refrigerator in small glass-stoppered bottles (125 ml.) under 3 ml. redistilled petroleum ether (BP 30-36°C.) to delay absorption of CO₂ from the air.
3. For alkaline phosphatase: To 25 ml. alkaline buffered substrate stock add 25 ml. distilled water. Make up daily as needed.
4. For acid phosphatase: 25 ml. buffered substrate stock is added to 25 ml. 0.1 N CH₃COOH. Mix well. Do not attempt to store.
0.1 N CH₃COOH. Weigh out enough glacial acetic acid to give 6.00 gm. of acid. Dissolve and dilute to 1000 ml. Mix well.

Method:

Alkaline Phosphatase:

1. Prepare a reagent blank and two standards as outlined in Steps 4 and 5 in Inorganic Phosphorus p. 225.

Procedure for control sample:

1. Pipet 5.0 ml. dilute alkaline substrate into glass-stoppered bottle.
2. Add 4.5 ml. 10% CCl₃COOH.
3. Add 0.5 ml. serum, stopper. Mix and filter.
4. Pipet 5 ml. of the filtrate into a cuvet.

Procedure for enzyme sample:

1. Pipet 5.0 ml. dilute substrate into glass-stoppered bottle.
2. Place in incubator to reach 37° C.
3. Add exactly 0.5 ml. serum. Mix, note time.
4. Remove at end of one hour.
5. Immediately add 4.5 ml. 10% CCl₃COOH.
6. Mix and filter.
7. Pipet 5.0 ml. of the filtrate into a cuvet.

Development of color:

Add 1.0 ml. of molybdate II, mix. Add 4.0 ml. of dilute pictol, mixing each cuvet immediately after the addition.

Photometry:

Set blank at 100 and read control sample and enzyme sample and standards, after 10 minutes and before 60 minutes using 680 mu or a red filter.

Calculation:

$$(D_u/D_s) C_s \frac{100}{0.25} = \text{mg. P/100 ml. serum (calculated as serum phosphorus).}$$

One phosphatase unit will liberate one mg. of inorganic phosphate from glycerophosphate in one hour under the specified conditions.

P_e = mg. P/100 ml. serum in incubated sample.

P_c = mg. P/100 ml. serum in unincubated sample (control).

$P_e - P_c$ = phosphatase units/100 ml. serum

Example:

P_e = 8.0 mg. %

P_c = 3.3 mg. %

Phosphatase = 4.7 Bodansky units/100 ml. serum. (8.0 - 3.3).

Precaution:

1. As for inorganic phosphate.
2. Exact time and temperature control is important.

Acid Phosphatase:

Procedure: As in alkaline phosphatase except using the acid substrate. Use female serum control for check of the method.

Interpretation:

Phosphorus: Normal values--5 mg. % in infants and children
3.7 mg. % in adults

In severe nephritis P may increase to 15-20 mg. %. In rickets P may fall to 2 mg. % or lower. Insulin injection decreases P in serum due to formation of hexose phosphates.

Phosphatase:

Bodansky units

Alkaline: Normal values 5-12 units in children
1.5-4.0 units in adults

Increased in Paget's disease (up to 50x)

Rickets (up to 20x)

Hyperparathyroidism (up to 10X)

Infectious Hepatitis (2-4X)

Other hepatic diseases (2-10x)

Acid: Normal values 0.0 - 1.1 units

Elevated only in 15-50% of cases of prostatic carcinoma with metastases (up to 30 units).

Erythrocytes contain an acid phosphatase so hemolysis should be avoided.

PHOSPHORUS - Lipid

Serum

Principle:

Total lipids are extracted as in the cholesterol procedure. The alcohol-ether extract is dried, and digested with sulfuric acid and hydrogen peroxide. The color is developed as under the inorganic phosphorus procedure.

Procedure:

1. Prepare an alcohol-ether extract of serum as outlined under Cholesterol, p. 152.
2. Pipet 10.0 ml. of extract into a 25 x 200 mm. test tube graduated at 25.0 ml.
3. Evaporate to dryness using a hot water bath, avoid the use of an open flame.
4. Add 2.5 ml. 5 N H_2SO_4 and a small quartz chip and digest until the water evaporates and the mixture turns brown or black.
5. Allow to cool and add 1 drop of 30% H_2O_2 directly into the mixture.
6. Continue heating. Repeat H_2O_2 treatment and reheating until colorless.
7. Cool the tube, add a few ml. of water, heat to boiling. Cool immediately, dilute the contents to 25.0 ml. Mix well.
8. Transfer 5.00 ml. to a photometer cuvet.
9. Proceed as in Inorganic Phosphorus, p. 224.

Calculations:

Since 0.4 ml. of serum is represented by 10.0 ml. of filtrate

$$C_u = (D_u/D_s) C_s \times \frac{100}{0.4}$$

$$= (D_u/D_s) C_s \times 250 = \text{mg. lipid P/100 ml. serum}$$

PORPHOBILINOGEN - Qualitative Urine

Reference:

Watson, C. J. and S. Schwartz. A simple test for urinary porphobilinogen.
Proc. Soc. Exp. Biol. & Med. 47, 393-4 (1941)

Principle:

Porphobilinogen in the urine forms a red colored compound with Ehrlich's Reagent in acid solution. This colored compound is insoluble in chloroform.

Reagents:

1. Saturated sodium acetate solution.
2. Ehrlich's Reagent. (Add 0.7 grams of p-Dimethylaminobenzaldehyde to 150 ml. of concentrated HCl and 100 ml. of water.)
3. Chloroform.

Procedure:

1. To 2 ml. of urine in a test tube add 2 ml. of Ehrlich's reagent.
2. Invert until mixed. Allow to stand 5 minutes.
3. Add 4 ml. of saturated sodium acetate to the tube and invert until mixed.
4. Note the color, if any.
5. Add 1 ml. of chloroform and shake vigorously. Allow to stand until separated.
6. Note the color of the chloroform layer.
7. If a pink to red color develops in step four, porphobilinogen and/or urobilinogen may be present. If in step six the chloroform layer is red, while the urine layer becomes colorless or the original color of the urine, then porphobilinogen is absent and urobilinogen is present. If the chloroform layer remains colorless and the urine layer is pink to red, then urobilinogen is absent and porphobilinogen is present. If both layers are red, then both porphobilinogen and urobilinogen is present. If large amounts of urobilinogen are present, the chloroform extraction may have to be repeated several times to remove all of the urobilinogen red. If both layers are colorless, then both porphobilinogen and urobilinogen are absent.
8. Report porphobilinogen and urobilinogen as negative or positive.

Notes:

Normal red-brown urines tend to disguise small amounts of pink color in the urine layer.

Interpretation:

In acute porphyria the urine contains porphobilinogen which gives a positive Ehrlich reaction. Positive results may also be obtained in cases of liver disease. Urobilinogen is normally found in the urine as it partially escapes absorption by the liver and is excreted by the kidneys.

PORPHYRIN DETERMINATION

Qualitative and Quantitative

References:

- Askevold, R.: Routine analysis of porphyrins in urine. *Scandinavian J. of Clin. and Lab. Invest.* **3**, 318-9 (1951).
- Brunsting, L.K., H.L. Mason and R.A. Aldrich: Adult form of chronic porphyria with cutaneous manifestations. *JAMA* **146**, 1207-12 (1951).
- West, E.S. and W.R. Todd: *Textbook of Biochemistry*, 2nd ed. (MacMillan Co., New York) 485-7 (1955).
- Gradwohl, R.B.H.: *Clinical Laboratory Methods and Diagnosis*, 5th ed. (C.V. Mosby Co., St. Louis) 551-3 (1956).

There are present in the excreta of the human body, pigments which have been identified as porphyrins. These pigments are closely related to the protoporphyrin found in the hemoglobin molecule, but are chemically distinct from it. In many diseases abnormal amounts of these pigments are excreted in the urine and feces. Therefore, a determination of the type and quantity of porphyrin present in the patient can be a valuable diagnostic tool.

A large variety of porphyrins are known to exist, some occurring naturally, while others have been synthesized. Of these the coproporphyrins and the uroporphyrins have been found to be particularly clinically significant with respect to certain diseases. A further specificity is observed in the isomers of both of these compounds; i.e., coproporphyrin I, coproporphyrin III, uroporphyrin I and uroporphyrin III have been found to be particularly related to a number of specific diseases.

A qualitative method for determining the presence of porphyrin, and a quantitative method for determining the amount of porphyrin present are here described.

Qualitative Porphyrin Determination

Principle:

In many cases a qualitative test is sufficient to indicate the presence of an abnormal quantity of porphyrins in the urine since the minute amounts that are normally present will not react to the test. Once the presence of the porphyrins has been established a quantitative test is run to determine the degree of abnormality.

The qualitative determination of the presence of porphyrins is based on a primary fluorescence in the near ultraviolet range of the spectrum exhibited by porphyrins in solution. However, since the fluorescence is easily masked by urinary impurities, the porphyrins must be separated from the original sample. This is accomplished by extraction, with a normal butyl alcohol-ethyl acetate mixture, from an acid aqueous solution.

Should the test be positive, a further extraction with ether will separate the uroporphyrins and the coproporphyrins, permitting a more specific identification. Both exhibit a characteristic fluorescence in the near ultraviolet range.

Apparatus:

1. An ultraviolet light emitting in the near ultraviolet range of the spectrum.
2. 50 ml. or 100 ml. separatory funnels.

Reagents:

1. Glacial acetic acid.
2. 1:1 (v/v) mixture of n-butyl alcohol and ethyl acetate.
3. 10% NaOH.
4. Concentrated HCl.
5. Ethyl ether.

Procedure:

1. Acidify a 15 ml. fresh urine sample with 3 ml. glacial acetic acid.
2. Shake the acidified urine in a separatory funnel with an equal volume of the n-butyl alcohol-ethyl acetate mixture. Discard the aqueous phase.
3. Wash the organic phase three times with distilled water.
4. The organic phase will exhibit a red fluorescence under the ultraviolet light if porphyrins are present. If the red fluorescence is evident at this point proceed with the rest of the determination to determine which of the porphyrins are present.
5. Extract the porphyrins from the organic phase with three portions (3 to 5 ml.) of 10% NaOH solution. The porphyrins go into the NaOH solution almost quantitatively. Discard the organic phase.
6. Neutralize the NaOH solution with concentrated HCl until the solution turns Congo Red paper gray.
7. Add 1 to 2 ml. glacial acetic acid.
8. Shake the solution with an equal volume of ether, in a separatory funnel. Coproporphyrins will transfer to the ether phase while the uroporphyrins will remain in the aqueous phase.
If large amounts of porphyrins appear to be present, the aqueous phase may be extracted several times with ether to insure the removal of the coproporphyrins.
9. Acidify the aqueous phase with HCl and examine for red fluorescence under light in the near ultraviolet range.
10. Extract the coproporphyrins from the ether phase with HCl. Examine the aqueous phase under the ultraviolet light. If coproporphyrins are present a red fluorescence will appear.
11. The coproporphyrins and uroporphyrins are reported as positive or negative.

Note: The qualitative procedure is summarized in Table I.

Table I
Qualitative Determination of Porphyrins

15 ml. urine plus 3 ml. glacial acetic acid; add n-butyl alcohol-ethyl acetate mixture; separate organic and aqueous phases.

Aqueous phase	Organic phase	
	Wash with distilled water, three times	
Discard	Wash water	Washed organic phase. Extract with 10% NaOH
	Discard	Extracted organic phase
		Alkaline aqueous phase. Neutralize with HCl; add 1-2 ml. glacial HAC; extract with ether.
		Discard
		Aqueous phase
		Acidify with HCl. Fluorescence indicates uroporphyrins
		Organic phase
		Extract with HCl; discard organic phase; red fluorescence in aqueous phase indicates coproporphyrins.

Quantitative Porphyrin Determination

Principle:

Once the presence of the porphyrins has been established, the amounts present must be determined. This is done spectrophotometrically for both the coproporphyrins and the uroporphyrins, following isolation from sources of interference in the original sample, and from each other. The coproporphyrins (I and II) are separated from the urine sample by extraction from the aqueous media with ether containing acetic acid. Uroporphyrin III is extracted from the aqueous media, at a pH of 3.0-3.2, with ethyl acetate. (Authentic uroporphyrin I is not extractable by ethyl acetate.)

The maximum light absorption of the coproporphyrins has been found to occur at a wave length of 401 mu while that of the uroporphyrins has been established at 405 mu. Thus a determination of the optical density of a solution containing the isolated porphyrin will permit calculation of the porphyrin concentration in the original sample.

Apparatus:

1. Any spectrophotometer reading in the 380-430 mu range is satisfactory, although the Beckman DU is recommended.
2. Separatory funnels.

Reagents:

1. Glacial acetic acid
2. 1.0 N acetic acid
3. 1.0 N sodium acetate
4. Ethyl ether
5. 3% (wt./vol.) CaCl_2 solution
6. 5% (wt./vol.) Na_2HPO_4 solution
7. 1.0 N NaOH
8. 0.1 N NaOH
9. 0.5 N HCl
10. 0.1 N HCl

Coproporphyrin Procedure:

1. Acidify 15 ml. of urine with 1 ml. of glacial acetic acid.
2. Extract the acidified sample in a separatory funnel, with 30.0 ml. of ethyl ether. Discard the aqueous layer.
3. Wash the ether layer, till clear, with distilled water.
4. Extract the coproporphyrins from the ether with five 1 ml. portions of 0.1 N HCl.
5. The total volume of acid extract will be very close to 5 ml.
6. On the spectrophotometer, determine the optical density using 1.0 cm. cuvetts of the solution at 380, 401 and 430 mu against a 0.1 N HCl blank.

Coproporphyrin Calculations:

From the determined optical densities the total micrograms in the sample, and the microgram per cent are determined. In equation (1) D_{corr} (optical density corrected for absorption due to impurities in the porphyrin solutions) is calculated.

$$(1) \quad \frac{2D_{401} - (D_{380} + D_{430})}{1.833} = D_{\text{corr}}$$

where D = optical density. $D_{\text{corr}} = 0.65$ corresponds to 1 microgram of coproporphyrin per ml. Therefore,

$$(2) \quad \frac{D_{\text{corr}}}{0.65} = \text{micrograms coproporphyrin/ml. acid extract}$$

Then, the total micrograms of coproporphyrin present in the acid extract is

$$(3) \quad \frac{D_{\text{corr}}}{0.65} \times \text{ml. acid extract} = \text{micrograms coproporphyrin}$$

This is equivalent to the total micrograms of coproporphyrin present in the original urine sample. The microgram per cent is then determined by dividing the total micrograms present by the original sample size, and multiplying by 100.

$$(4) \quad \frac{D_{\text{corr}}}{0.65} \times \frac{\text{ml. acid extract}}{\text{ml. sample}} \times 100 = \text{microgram per cent coproporphyrin}$$

Since, under normal conditions, the ml. acid extract = 5 and the ml. sample = 15, the formula may be simplified to

$$(5) \quad \frac{D_{\text{corr}}}{0.65} \times 33.3 = \text{microgram per cent coproporphyrin}$$

The total urine volume, total micrograms coproporphyrin and the total microgram per cent coproporphyrin are reported.

Uroporphyrin Procedure:

1. Bring a 5 ml. urine sample to a pH of 5.0 to 5.5 with 5 ml. of acetate buffer. (Check with pH paper.)
2. Place 2 ml. of the mixture in a centrifuge tube; add 2 drops of phosphate solution, 2 ml. CaCl_2 solution and 2 ml. 1.0 N NaOH. Mix well.
3. Centrifuge at 1500 rpm for 10 minutes. Discard the supernatant liquid.
4. Wash the precipitate with 0.1 N NaOH, centrifuge, and decant supernatant liquid.
5. Wash twice with distilled water, centrifuging and discarding supernatant liquid each time. A red fluorescence (indicating porphyrins) under an ultraviolet light should not be present at this stage. If this occurs the unprecipitated porphyrins may be precipitated by the addition of more CaCl_2 solution.

6. Dissolve the precipitate in 10.0 ml. of 0.5 N HCl.
7. The optical density of the solution is read at wavelengths of 380, 405 and 430 mu.; 0.5 N HCl is used as the blank.

Uroporphyrin Calculations:

The uroporphyrin calculations follow the same pattern as those for the coproporphyrin. Thus, the following steps are used in calculating the total micrograms of uroporphyrin and the total microgram per cent.

$$(1) \quad \frac{2 D_{405} - (D_{380} + D_{430})}{1.844} = D_{\text{corr}}$$

$$(2) \quad \frac{D_{\text{corr}}}{0.65} \times \text{ml. acid solution} = \text{total micrograms uroporphyrin per ml. of original sample}$$

Since only 1 ml. of the original sample was used:

$$(3) \quad \text{Total micrograms uroporphyrins} \times 100 = \text{microgram per cent uroporphyrins}$$

The total urine volume, total micrograms uroporphyrin and the total microgram per cent uroporphyrin should be reported.

Interpretation:

The normal amounts of coproporphyrin and uroporphyrin excreted daily is small, 50-300 micrograms and 0.5 micrograms respectively. However, in certain disease conditions abnormal amounts of these porphyrins appear.

Coproporphyrin I and III occur normally in human urine in minute and approximately equal amounts. But, in a variety of diseases the excretion of these porphyrins is greatly increased. Also, the ratio of the I to the III isomer may vary greatly, depending upon the disease condition. For example, in poliomyelitis and lead poisoning coproporphyrin III is excreted while in infectious hepatitis coproporphyrin I predominates.

The excretion of uroporphyrins is characteristic of the metabolic disease of porphyria. The photosensitive or congenital type is characterized by the excretion of uroporphyrin I while acute porphyria is characterized by the excretion of uroporphyrin III.

Exact and reliable data relating porphyrin concentration to specific diseases conclusively is not available. However, the abnormality of the porphyrin concentration, when coupled with other symptoms will help complete the diagnostic picture.

**PROTEIN - (Turbidimetric)
Spinal Fluid and Urine**

References:

1. Looney, J.M. and Walsh, A.I.: J. Biol. Chem. **127**, 117 (1939).
2. Henry, R.J., Sobel, C., and Scaglove, M.: Proc. Soc. Exp. Biol. Med. **92**, 748 (1956).

Principle:

Protein in spinal fluid or urine is precipitated by trichloroacetic or sulfosalicylic acid as a fine uniform suspension. The turbidity thus produced is measured photometrically.

Reagents:

- Sulfosalicylic acid, 3 per cent solution or
- Trichloroacetic acid, 3 per cent solution
- (Keep refrigerated)

Procedure:

1. Into a test tube place 1.0v ml. of cerebrospinal fluid. Add 4.0 ml. of 3% sulfosalicylic acid and immediately mix by inverting several times.
2. Allow to stand at room temperature for five minutes and read before 10 minutes against a blank of distilled water using light of about 660 mu (red).

Calculation:

Interpolate on a calibration curve or chart.

Standardization:

Prepare dilutions of a previously standardized serum containing from 0.1 to 3 mg. of protein per ml. of solution. Carry out the procedure above and plot the results.

Notes:

1. Since the degree of reproducibility depends upon many physical factors such as temperature, speed of mixing, etc., the details of the procedure should be standardized as much as possible.
2. Trichloroacetic acid produces more turbidity from globulins than from albumin (1.2 to 1) while sulfosalicylic acid produces more turbidity from albumin (2 to 1) (Ref. 2). For this reason a mixture of the two acids, 2 of sulfosalicylic to 4 of trichloroacetic has been recommended.
3. The wavelength of 660 mu is advocated because any yellow color of spinal fluid or urine will not affect the optical density at this wavelength.
4. At best, the degree of reproducibility is no better than $\pm 10\%$ and at times as high as $\pm 20\%$.

Interpretation:

See p. 239 (spinal fluid)

PROTEIN - TOTAL AND ALBUMIN/GLOBULIN RATIO

Reference:

Gornall, Allen G., Bardawell, C. J., and David, M. M.; J. Biol. Chem. **177**, 751 (1949).

Principle:

Serum proteins (total) and the albumin fraction (after globulin precipitation) are reacted with alkaline copper reagent (biuret reagent) to give a bluish-purple color which is measured photometrically in the presence of the blue color of the excess copper.

Apparatus:

Matched test tube cuvetts 19 x 150 mm. or 19 x 105 mm.

Reagents:

1. Biuret reagent: Into a 1000 ml. volumetric flask place:

$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1.5 g.

$\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4\text{H}_2\text{O}$ (Rochelle Salt) 6.0 g.

1.0 g. KI (optional)

Dissolve in approximately 500 ml. Add and mix rapidly 300 ml. 2.5 N NaOH prepared from concentrated NaOH; see stock reagents).

Add water and mix to make 1000 ml.

2. Sodium sulfate 22.6%. Dissolve 226 g. of the best quality anhydrous Na_2SO_4 in water to make 1000 ml. of solution.

3. Ethyl ether

4. Sodium Chloride 1%

Procedure:

Total Protein:

Into a dry test tube which can be centrifuged, measure 0.50 ml. of serum, and add 10.0 ml. of 22.6% Na_2SO_4 solution. Stopper the tube, and mix thoroughly by inversion (not shaking). At once, transfer 2.0 ml. to photometer tube T for total protein.

Albumin ($\frac{1}{2}$ a₁ globulin)

To the remaining serum-sodium sulfate mixture, add 3 ml. of ether, shake vigorously for 30 seconds, and centrifuge while still stoppered; slant the tube, and transfer 2.0 ml. of the clear, supernatant aqueous phase to a dry photometer tube A for the albumin determination.

Blank:

Into a dry photometer tube B, place 2.0 ml. of 22.6% Na_2SO_4 solution.

To each of these three photometer tubes add 4.0 ml. of biuret reagent, and mix well. Allow the tubes to stand for 30 minutes at room temperature (20-25° C.).

Read in the photometer against the blank set at 100% transmission using wavelength 550 mμ. From the transmission in % determine the density (transmission vs. optical density chart) and refer this value to a calibration curve prepared as described below or multiply the density by a factor.

Procedure for total protein without albumin determination:

1. **Blank:** Into photometer tube **B** measure 2.0 ml. 1% NaCl solution.
2. **Total protein:** Into photometer tube **T** measure 2.0 ml. of 1% NaCl solution. Pipet into this 0.100 ml. of serum with a "to contain" micropipet.
3. To each of the photometer tubes add 4.0 ml. biuret reagent and mix well.

Let stand 30 minutes at 20-25 C. and read in photometer as above. Refer to the same calibration curve; since the volume in this case is 6.1 ml. instead of 6.0 ml. the final results for the simplified total protein should be multiplied by 61/60.

Calibration:

Pipet 5.00 ml. of clear normal (preferably pooled) serum into a 50 ml. volumetric flask, dilute to mark with 0.9% NaCl, and mix. Prepare in duplicate a series of nine photometer tubes as follows:

Tube #	0.9% ml. NaCl	(a) ml. dil. serum	(b) (V)	(c) (V x P)
1	2.00	0.00	0.00	0.00
2	1.85	0.15	0.1575	----
3	1.70	0.30	0.3150	----
4	1.55	0.45	0.4725	----
5	1.40	0.60	0.630	----
6	1.20	0.80	0.840	----
7	1.00	1.00	1.050	----
8	0.80	1.20	1.260	----
9	0.60	1.40	1.475	----

Develop color in each tube by adding 4.0 ml. biuret reagent as above.

- (a) Serum diluted 1 volume up to 10 volumes with 0.85% NaCl
- (b) Column (a) corrected for 1:21 instead of a 1:20 dilution of unknowns
- (c) Values to be plotted vs. optical density. "nominal" protein concentration.

P = protein concentration of the original serum as determined by

Kjeldahl procedure

Determine total nitrogen and non-protein nitrogen and multiply protein nitrogen by 6.25 to obtain protein in g. %.

The resulting graph gives a practical straight line and therefore a Beer's Law factor may be used with a maximum error of 0.1-0.2 g. % protein.

Notes:

1. For room temperatures above 25°C. - recalibrate.

Interpretation:

1. A variety of different precipitating salts have been used. With this present method albumin is usually 4.5 g. %; globulin 2-2.5g%; total protein 6-7.5 g. %. A total salt concentration higher than this gives lower "albumin" and higher globulin.

PROTEIN

Spinal Fluid - Biuret Method

Reference

1. Rosenthal, H. L. and Cundiff, H. T.; Clin. Chem. 2, 394 (1956)

Principle:

Ethylenediamine tetraacetate is added to the biuret reagent (in place of tartrate) to avoid turbidities due to calcium when the necessarily larger volumes of spinal fluid are used.

Reagents:

1. Stock protein standard

A standardized serum (or serum albumin) is accurately diluted with 0.85% NaCl to about 50 mg. protein per milliliter. This may be stored about 30 days at 4°C. or indefinitely at -20°C.

2. Protein working standard

Dilute 5.00 ml. protein stock solution to 100 ml. with 0.85% NaCl. Prepare on day of use.

3. EDTA-biuret reagent

Dissolve 1.50 g. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in about 500 ml. distilled water. Add 6.0 g. disodium ethylene diamine tetraacetate (Na_2EDTA) and 1.0 g. KI and dissolve. Add 300 ml. 2.5 N NaOH, mix well and dilute to one liter. Store at room temperature in polyethylene containers.

Procedure:

Blank - 2.00 ml. 0.85% saline

Standard - 2.00 ml. protein working standard

Unknown - 2.00 ml. cerebrospinal fluid

Add 4.00 ml. of EDTA-biuret reagent and mix well.

Allow to stand 20-30 minutes at room temperature, and then compare standard and unknown against the blank in a photometer set at 550 mμ.

Calculation:

$$\left(\frac{D_u}{D_s}\right) C_s \times \frac{100}{2} = \text{mg. protein/100 ml. spinal fluid}$$

$$C_s = \text{mg. protein present in 2.00 ml. of protein working standard.}$$

Notes:

1. A series of standard protein solutions may be used to prepare a standard curve instead of using daily standards.
2. If the protein value exceeds 300 mg./100 ml. the sample should be diluted and the analysis repeated.
3. If desired, the same reagent may be used for serum protein analysis; however, the sensitivity of the reagent is somewhat less.

Interpretation:

Normal - lumbar spinal fluid - 15-40 mg.% total protein - almost entirely albumin
Globulins may be detected by the Pandy test,

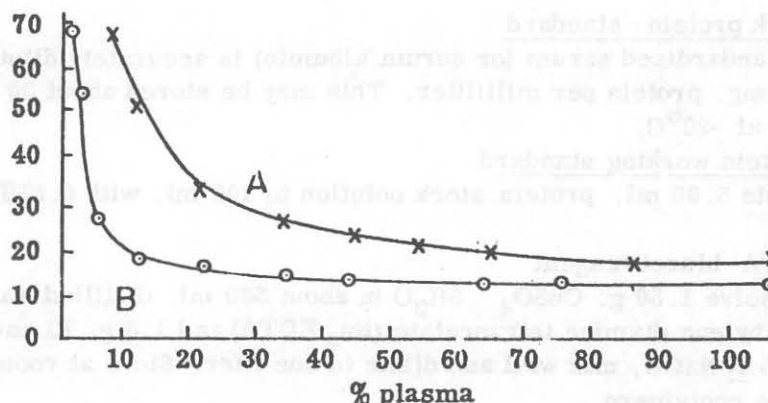
PROTHROMBIN TIME (One-stage Quick Method)

Reference:

Quick, A. J., Am. J. Clin. Path. 10, 222 (1940)

Principle:

Oxalated plasma is treated with an excess of thromboplastin and an optimum concentration of Ca^{++} . The clotting time is determined and related to the pro-thrombin content of the plasma, as shown in the accompanying representative graph (A) of clotting times vs. plasma concentration (in 0.85% saline).



Obviously dilution of plasma in saline (curve A) causes a dilution of not only prothrombin but also of all other clotting factors, except thromboplastin and Ca^{++} which are kept constant. If normal plasma is diluted in plasma previously treated by BaSO_4 (which removed pro-convertin and prothrombin) and clotting times again determined curve B is obtained. The difference between the two curves represents the combined effect of factors other than prothrombin and proconvertin. Curve B is the combined dilution curve of prothrombin and proconvertin.

Since in dicoumarol and hedulin therapy for thrombosis both prothrombin and proconvertin are decreased, curve B represents the usual therapeutic situation.

The only other factor likely to affect the results is pro-accelerin and since this is labile, collection problems and delay in analysis may affect this markedly. This factor can be added in excess by the use of BaSO_4 plasma.

In this laboratory we run both the regular Quick one-stage procedure and the regular procedure modified by the addition of BaSO_4 plasma. We call these tests "uncorrected" and "corrected". The "corrected" test is run only when pro-accelerin deficiency or lack is indicated.

Reagents:

1. Plasma. By careful venipuncture collect exactly 4.5 ml. of whole blood. Mix in centrifuge tube with exactly 0.5 ml. of 0.1 molar sodium oxalate (1.34 g. of anhydrous sodium oxalate in 100 ml.). Centrifuge at once for

4 minutes at 2,000 r.p.m. Pipet off the supernatant plasma, avoiding any re-mixing of red blood cells. Use within a short time. If there is to be a delay before determining prothrombin activity, keep plasma chilled until it is to be used. Warm chilled plasma in water bath for 5 minutes before use, or 15 minutes in warm room.

2. Thromboplastin.

3. 0.0125 M CaCl_2 (calcium chloride).

4. Dilute pro-accelerin plasma (see Preparation of Reagents below).

Procedure:

1. Quick procedure ("Uncorrected") - Warm all reagents to 37° C. before use. Conduct tests at 37° C.

a. Using 0.1 micro-pipets, mix 0.1 ml. of thromboplastin and 0.1 ml. of calcium chloride solution in a small test tube, approximately 10 x 75 mm. Keep in water bath or air bath, at 37° C.

b. Using 0.1 ml. micro-pipet, blow 0.1 ml. of warmed plasma into the 0.2 ml. of thromboplastin-calcium mixture. Mixing of reagents should be rapid and complete.

c. Start timer simultaneously with addition of plasma.

d. Stop timer when end-point is reached. The end-point is the first appearance of a clot as determined by visual observation while (1) tapping the tube with a finger, (2) tilting the tube, or (3) drawing a loop of #20 nichrome wire through the coagulation mixture or (4) by a photoelectric recorder.

e. The prothrombin time thus obtained has no absolute meaning but must be converted into terms of prothrombin activity by reference to a previously established normal plasma dilution curve, or a table prepared from it (see below).

2. Quick procedure ("Corrected") - Using 0.1 ml. and 0.2 ml. pipets mix 0.2 ml. thromboplastin-calcium and 0.1 ml. of dilute pro-accelerin plasma, incubate at 37° C. for 5 minutes. Then add 0.1 ml. of the test plasma and start timing by one of the methods listed above.

Notes and Interpretation:

The determination of the blood prothrombin level is of great importance in the diagnosis of hemorrhagic states due to Vitamin K deficiency, biliary obstruction and liver damage. The hypoprothrombinemia produced by dicoumarol in the prophylaxis and therapy of intravascular thrombosis requires frequent prothrombin determinations in order to guard against spontaneous hemorrhage.

The optimal concentration of calcium required for prothrombin determination is influenced by the amount of oxalate used in collecting the blood sample. This is especially true for the one-stage method. Quick's directions for oxalating 4.5 ml. of whole blood with 0.5 ml. of 0.1 molar sodium oxalate must be adhered to rigidly. Some laboratories use citrate or EDTA. When changes are made, the optimal Ca^{++} concentration must be redetermined. The fibrinogen level of plasma is usually of little consequence

PROTHROMBIN TABLE

Values in the body of the table represent the ratio between
the clotting times of the unknown to the control plasma $\frac{U \text{ sec.}}{C \text{ sec.}}$

C sec.

[illegible]

calculation: (using ratio method)

1. Calculate the ratio Unknown time (sec.)/Control time (sec.)
2. Find value closest to this ratio in the table.
3. Read prothrombin % directly.

Example: Control time = 16.3 sec. Uncorrected
 = 15.2 sec. Corrected
 Unknown time = 17.2 sec. Uncorrected
 = 15.8 sec. Corrected

Uncorrected	Corrected
1.00	1.00
0.99	0.99
0.98	0.98
0.97	0.97
0.96	0.96
0.95	0.95
0.94	0.94
0.93	0.93
0.92	0.92
0.91	0.91
0.90	0.90
0.89	0.89
0.88	0.88
0.87	0.87
0.86	0.86
0.85	0.85
0.84	0.84
0.83	0.83
0.82	0.82
0.81	0.81
0.80	0.80
0.79	0.79
0.78	0.78
0.77	0.77
0.76	0.76
0.75	0.75
0.74	0.74
0.73	0.73
0.72	0.72
0.71	0.71
0.70	0.70
0.69	0.69
0.68	0.68
0.67	0.67
0.66	0.66
0.65	0.65
0.64	0.64
0.63	0.63
0.62	0.62
0.61	0.61
0.60	0.60
0.59	0.59
0.58	0.58
0.57	0.57
0.56	0.56
0.55	0.55
0.54	0.54
0.53	0.53
0.52	0.52
0.51	0.51
0.50	0.50
0.49	0.49
0.48	0.48
0.47	0.47
0.46	0.46
0.45	0.45
0.44	0.44
0.43	0.43
0.42	0.42
0.41	0.41
0.40	0.40
0.39	0.39
0.38	0.38
0.37	0.37
0.36	0.36
0.35	0.35
0.34	0.34
0.33	0.33
0.32	0.32
0.31	0.31
0.30	0.30
0.29	0.29
0.28	0.28
0.27	0.27
0.26	0.26
0.25	0.25
0.24	0.24
0.23	0.23
0.22	0.22
0.21	0.21
0.20	0.20
0.19	0.19
0.18	0.18
0.17	0.17
0.16	0.16
0.15	0.15
0.14	0.14
0.13	0.13
0.12	0.12
0.11	0.11
0.10	0.10
0.09	0.09
0.08	0.08
0.07	0.07
0.06	0.06
0.05	0.05
0.04	0.04
0.03	0.03
0.02	0.02
0.01	0.01
0.00	0.00

<u>Uncorrected</u>	<u>Corrected</u>
--------------------	------------------

$$\frac{17.2}{16.3} = 1.056 = -85\% \qquad \frac{15.8}{15.2} = 1.040 = 89\%$$

$$\frac{17.2}{16.3} = 1.056 = -85\% \qquad \frac{15.8}{15.2} = 1.040 = 89\%$$

is realized that these "percentages" of normals do not actually represent the patient's prothrombin concentration. However, clinicians are accustomed to treating patients on the basis of this dilution curve of Dr. Quick and we continue to use it as a matter of convenience. The more accurate procedures are also somewhat more time consuming.

TABLE FOR DETERMINING PROTHROMBIN PERCENTAGES

(Manual of Clinical Biochemistry - W. G. Karr,

J. G. Reinhold, and F. W. Chernock - p. 49)

Control		Prothrombin, Per cent of Control							
100	85	70	60	50	40	30	20	10	6
Seconds									
12.5	13.2	14	14.8	16	18	21	26	38	70
13.0	13.7	14.5	15.4	16.6	18.7	21.8	27	40	73
13.5	14.3	15.1	16.0	17.3	19.4	22.7	28	41	76
14	14.8	15.7	16.6	18	20.2	23.5	29	43	78
14.5	15.3	16.3	17.2	18.6	20.9	24.4	30	44	81
15	15.8	16.8	17.8	19.2	21.6	25	31	46	84
15.5	16.4	17.4	18.4	19.8	22.3	26	32	47	87
16	16.9	17.9	19	20.5	23	27	33	49	90
16.5	17.4	18.5	19.5	21.1	23.8	27.7	34	50	92
17	18	19	20.1	21.8	24.5	28.6	35	52	95
17.5	18.5	19.6	20.7	22.4	25.2	29.4	36	53	98
18	19	20.2	21.3	23	26	30.2	37.5	55	101
19	20	21.3	22.5	24.3	27.5	31.9	39.8	58	106
20	21	22.4	23.7	25.6	29	33.6	42	61	112
21	22.2	23.5	24.9	26.9	30.2	35.3	44	64	118
22	23.2	24.6	26	28	31.7	37	46	67	123
23	24.3	25.8	27.2	29.4	33.1	39	48	70	129
24	25.4	26.9	28.4	31	34.6	40	50	73	135

Calculation:

Example: Normal Control time is 14.5 seconds. Patients time is 18.0 seconds.

Then prothrombin % is between 60 and 50%. By interpolation:

$$\frac{18.6-18.0}{18.6-17.2} = \frac{0.6}{1.4} = 0.428; (0.428 \times 10) \div 50 = 54.3\%$$

in the one-stage method, since considerable range of fibrinogen content appears to have little effect on the prothrombin time.

The lability of accelerator-globulin (labile factor) (accelerin or pro-accelerin) is frequently a source of error in the one-stage method. The handling of the plasma in this method has a distinct effect on the adequacy of its accelerator-globulin content. For this reason, the blood sample should not be exposed to extremes of centrifugation, either in intensity or duration. A short, light centrifugation is essential for the preservation of accelerator-globulin activity. Since accelerator-globulin is heat-labile, the plasma must not be incubated for long periods of time. Plasma should be tested for prothrombin activity within a short time after its collection or else should be chilled until needed for use.

The final factor required for prothrombin activity determination is thromboplastin. This factor must be present in excess. To a large extent the validity of the measurement of prothrombin activity is dependent upon the thromboplastin. The principal requirement for a satisfactory thromboplastic material is that it yield accurate and consistent prothrombin times. When all other factors have been controlled by careful, uniform technique, the prothrombin times obtained measure prothrombin activity only when the thromboplastin used gives reliably reproducible values for given prothrombin concentrations. Other requirements of a good thromboplastin are stability and convenience.

Proper handling of thromboplastin requires only a few precautions. Although thromboplastin is stable at room temperature for at least two weeks, it is advisable to store the material in the refrigerator at about 4° C. when not in use. The usual routine is to withdraw from the stock bottle a volume of thromboplastin sufficient for immediate requirements and to replace the stock bottle in the refrigerator. Care must be taken, however, to avoid freezing the solution.

If several prothrombin determinations are to be made, it is more convenient to mix equal volumes of thromboplastin and calcium chloride solution. 0.2 ml. portions of this thromboplastin-calcium mixture are pipetted into the required number of tubes and kept in the water bath ready for use. The test is then continued as described below. Discard unused mixture of thromboplastin-calcium at end of the working day. The order of addition of reagents as given in the directions must be followed. Changing the order by mixing plasma and thromboplastin and then adding calcium chloride solution sometimes gives erratic results, particularly if the mixture is incubated for any length of time. Reproducibility of accurate prothrombin times depends on uniformity of a technique. Only by careful observance of uniform procedure in the performance of the test can maximum accuracy be obtained.

Prothrombin times are used clinically primarily for control of anti-coagulant therapy in patients who have (a) phlebitis (b) coronary occlusion (c) any other disease which tends to produce intra-vascular clotting. The prothrombin level should be kept between 15% and 20% of normal although different clinicians and hospitals vary. Bleeding tendencies begin to show up at 10% or below in many patients.

PREPARATION OF PLASMA PROTHROMBIN REAGENTS

Preparation of proaccelerin plasma:

1. **Collection of plasma:** Collect by the use of vacutainer tubes by heart puncture more than 100 ml. of oxalated plasma with the oxalate concentration recommended by Quick (ref. cit.).
2. **Test for the presence of prothrombin and proconvertin** by the following tests, which also should be run after each adsorption (see (3).)

Tests for presence of prothrombin and/or proconvertin:

Test System A: 0.2 ml. thromboplastin-calcium

0.1 ml. rabbit plasma

Measure clotting time

Results:

In the original rabbit plasma the time may be as short as 6 seconds. When treated with BaSO_4 the time will be prolonged and should be over 150 seconds. However, this may not represent a complete removal of prothrombin but merely a removal of proconvertin since the latter is preferentially removed before prothrombin. Therefore Test System B must be used.

Test System B: 0.1 ml. aged human serum

0.2 ml. thromboplastin-calcium

Incubate 5 minutes

0.1 ml. rabbit plasma

Results:

The aged human serum contains no prothrombin (all has been converted to thrombin and adsorbed and neutralized) but does contain pro-convertin; now if the rabbit plasma is free of pro-convertin but still contains some pro-thrombin clotting will occur.

3. Add to 0.1 g. wet BaSO_4 (washed twice with distilled water by suspension and centrifugation) for each ml. of plasma, the volume of plasma obtained above. (1). Allow to stand at room temperature for 15 minutes resuspending the BaSO_4 at intervals. Centrifuge 10 minutes at 3000 r.p.m. (preferably in a refrigerated centrifuge) and carefully remove the supernatant plasma.

Note:

1. Treatment with washed BaSO_4 should be continued until the time of clotting in test system A and B are prolonged to more than 150 seconds.
2. Some samples of BaSO_4 are not very active in adsorption, and their activity may change with time. In this case, a new batch may be used or Quick's washed $\text{Ca}_3(\text{PO}_4)_2$ suspension may be used, or freshly prepared BaSO_4 may be used.
A. BaSO_4 To 8.5 ml. 1 M BaCl_2 add 8.5 ml. 1 M Na_2SO_4 (2 gm. BaSO_4). Mix, centrifuge, and resuspend in water to wash, recentrifuge. Repeat. Use the 2 gm. for 25 ml. of plasma. Allow to adsorb for 30 min. at room temperature.

B. $\text{Ca}_3(\text{PO}_4)_2$

Sol. A. 158 g. $\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$ in one liter H_2O

B. 66.6 g/ CaCl_2 anhyd. in one liter H_2O

Add Sol. A to Sol. B slowly and with vigorous stirring. Adjust pH to

7.0 by adding HCl or NaOH . Wash by decantation until free of Cl^- .

(AgNO_3 / KNO_3). Bring volume to 1 liter. This is a 0.02 M stock

suspension. For dilute suspension: Shake well. Dilute 4 ml. / 96 ml. H_2O .

For each ml. of plasma centrifuge 1 ml. of dilute suspension, and discard the supernate. Use as above.

3. The aged human serum is prepared as follows: Ten ml. of blood are drawn into a Vacutainer, which is then immediately opened. 0.2 ml. of thromboplastin (Soluplastin) is added and the blood mixed well. Clotting occurs rapidly and the tube is then allowed to sit at 37° for 30 - 40 minutes. The serum is then removed and is ready for the test. Usually a dilution of 1:10 or 1:5 with water can be made since the serum contains large amounts of convertin.

Test for Pro-accelerin Activity:

When the previous tests A and B have indicated the virtual absence of proconvertin and prothrombin from the rabbit plasma, its proaccelerin activity must be tested. For this we need a plasma free of or low in pro-accelerin. This is obtained by drawing a sample of blood into a prothrombin Vacutainer tube and mixing. Centrifuge and remove the plasma. Allow to remain at 37° until the "Uncorrected" Quick prothrombin time is prolonged to at least 30 seconds.

Then, using Test System C. determine how far the rabbit plasma (free of proconvertin and prothrombin) can be diluted and still retain its maximum activity.

Test System C.

0.2 ml. thromboplastin-calcium

0.1 ml. rabbit plasma

Incubate 5 min.

0.1 ml. aged human plasma

Usually dog plasma can be diluted at least 1:4 and rabbit plasma 1:8 before a noticeable decrease in activity is seen.

When the greatest dilution possible has been established, dilute the bulk of the rabbit plasma in water, mix well, adjust the pH to 7.4, and freeze in 2 ml. portions at -20°C . Use one or two portions daily. Do not refreeze.

Preparation for coagulation tubes:

After use, soak in a 2% solution of Na_2CO_3 5 hours or overnight. Rinse well with tap water and then with distilled water and bake at more than 110°C . overnight. If the tubes are siliconized, removal of the clots is somewhat simplified.

Directions for Siliconizing:

Silicone - One part Dri-Film (General Electric) #9987 is added to four parts Petroleum Ether.

Fill one tube with this mixture and pour into another rotating the first tube so that the entire inside area is covered. Avoid siliconizing the outside since the tubes will be too slippery to handle easily. Rinse the tubes (in a rack for convenience) with hot tap water, allowing the water to remain for about 10 minutes. This step removes any silicone flakes and speeds the removal of the HCl base of the dri-film. Rinse twice with distilled water and dry at 120°C , for 60-90 minutes.

To remove silicone - Put the tubes in a mixture of 2 l. distilled H_2O (in which 150 gms. NaOH is dissolved) and 2 l. of technical acetone. Allow to stand for at least 8 hours and rinse well after removing from the solution, dry at 120°C . Repeated removal of silicone by this solution will result in etched tubes that will not look clean, however, upon being resiliconized, the tubes will be entirely clear and the clots easily read. We suggest that you keep these tubes separate from the others.

PROTHROMBIN
(Prothrombin Consumption Test)
Serum

Reference:

Leon N. Sussman, M.D., Ira B. Cohen, M.D., and Robert Gittler, M.D.
J.A.M.A. 156, 673-752 (1954).

Technique:

Careful attention must be paid to details in technique, as minor variations produce very marked differences in the results obtained.

Preparation of Serum:

Blood is drawn cleanly and allowed to clot at room temperature. The blood is placed in a water bath at 37° C. for one hour after clotting. The blood is centrifuged for three minutes, and the serum is separated. Serum may be stored at 4° C. for a maximum of 60 minutes before the test.

Preparation of Reagents:

Thromboplastin solution is prepared as for the plasma prothrombin test. Fibrinogen solution is prepared as directed by the manufacturer (to contain 300 mg. per 100 cc. of fibrinogen and 85% of sodium chloride solution). Two cubic centimeters of thromboplastin solution and 1 cc. of fibrinogen solution are mixed just before the test.

Performance of Test:

The mixture of thromboplastin and fibrinogen, in the amount of 0.2 cc. is placed in a test tube in a water bath at 37° C. for five minutes. The serum to be tested is warmed in the water bath at 37° C. for five minutes. One-tenth cubic centimeter of the serum is blown strongly into the thromboplastin-fibrinogen mixture (the timer should be started simultaneously), and the time for the clot to form is determined as in the plasma prothrombin test.

The clot is formed as a fine web, and the person performing the test should watch carefully for it. There are other precautions to be observed. The serum must be refrigerated at 4° C. for a maximum of 60 minutes if it is not used immediately. The thromboplastin and fibrinogen must be mixed just before use. All reagents must be warmed at 37° C. for five minutes before use. The test is interpreted as follows: time longer than 30 seconds...normal range; time shorter than 20 seconds...abnormal range; and time between 20 and 30 seconds...doubtful.

Note:

BaSO₄ plasma entirely devoid of prothrombin and pro-convertin may be used instead of fibrinogen.

SALICYLIC ACID

Serum or Plasma

Reference:

Bernard B. Brodie, Sidney Udenfriend, and Alvin F. Coburn: *J. Pharmacol. Exp. Therapeutics* **80**, 114-117 (1944) (Modified).

Reagents:

Salicylic acid solution, stock standard, 100 mg. per 100 ml. Dissolve 116 mg. of sodium salicylate (U.S.P. cryst. Merck) in water and dilute to 100 ml. Mix. Stable when stored in refrigerator.

6 N HCl, made by diluting concentrated HCl with an equal volume of water.

Ethylene dichloride (Ethylene Chloride, Eastman #132).

1% $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ in 0.07 N HNO_3 . Dissolve 1 g. of ferric nitrate, A.C.S. in water. Add 0.44 ml. of concentrated nitric acid and dilute to 100 ml. Mix. This is a stock solution.

Dilute ferric nitrate solution. Add 3 ml. of above stock solution to 100 ml. of distilled water. Mix. Keeps a few days.

Sodium sulfate, reagent anhydrous.

Procedure:

Into a clean dry glass stoppered 60 ml. bottle place 1 ml. of serum or plasma. Add 0.2 ml. of 6 N HCl and 15 ml. ethylene dichloride. Shake 5 minutes, preferably by machine. Add approximately 1 g. anhydrous sodium sulfate. Shake 1 minute. Pour the organic layer into a tube, stopper with a cork, and centrifuge until clear.

Remove 10 ml. to another clean, dry bottle. Add 15 ml. of dilute ferric nitrate solution. Shake 5 minutes. Transfer 10 ml. of the aqueous layer to a cuvet. Read in a photometer (515 mμ) using distilled water for a blank.

Calculation:

Construct a standard curve by analyzing 1 ml. quantities of aqueous salicylate solution of varying concentrations, by the above procedure.

Interpretation:

Satisfactory plasma levels for treatment of rheumatic fever are 36 to 40 mg. % of salicylic acid. See Alvin E. Coburn: *Bull. Johns Hopkins Hosp.* **73**, 435-464 (1943). It is important to use the chemical analysis to control therapy, since the therapeutic dose is very close to the toxic level.

Note: (1) Save the ethylene dichloride solutions for recovery by distillation. (2) By this modification the absolute extraction of salicylic acid is only 95.8% complete but since standards and unknowns are treated in an identical manner, 100% recoveries are obtained.

SODIUM AND POTASSIUM Serum, Urine and Tissue

References:

1. Barnes, R. B., Richardson, D. Berry, J. W., Hood, R. L.: Ind. Eng. Chem. Anal. Ed., 17, 605 (1945).
2. Berry, J. W., Chappell, D. G., and Barnes, R. B., *ibid.*, 18, 19 (1946)

Principles of Flame Photometry:

In flame photometry, an aqueous solution is atomized under reproducible conditions by some sort of spraying apparatus. The vapor is conducted to the air intake of a gas burner and ignited. At the relatively low temperature of a gas burner, most elements are not energized to emission temperature and only sodium, potassium (and to a lesser degree calcium and magnesium) emit light.

The intensity of the light emitted is measured by a photocell with an appropriate filter combination (a prism or grating spectrophotometer may also be used to isolate the light wavelengths of interest). By measuring the intensity of light given by solutions containing known concentrations, a curve can be obtained which relates the instrument reading to concentration. This is known as the "Direct" method.

Many factors influence the intensity of light emitted and measured using a solution of constant composition. Some of these are listed.

1. Gas flow rate
2. Air flow rate
3. Temperature of flame
4. Rate of atomization
5. Efficiency of atomization
6. Presence of other ions (anions and cations)
7. Efficiency of filters, or of the prism or grating used to isolate wavelengths of light.
8. Stability of light measurement system (photocells) amplifier, and galvanometer
9. Purity of air supply (if burner supply is atmospheric).

Some of these factors can be controlled, some are inter-related, others may vary erratically or are uncontrollable. The "Internal Standard" was developed to minimize the effect of variation in these factors.

In the "Internal Standard" procedure, a known concentration of a salt containing an element with an emission line different from the element being determined, is added to the standard and to the unknown series of solutions. Lithium salts are commonly used. In this method the lithium emission is affected to the same extent as that of the element being determined by most of the foreign influences listed above. Instead of measuring direct intensities, the ratio of the intensities of light produced by (e.g.) sodium to lithium is measured, and the ratio is not affected by the variable factors, 1-6.

Reagents:

1. Stock lithium solution 2 N
LiCl 84.80 g./liter
or LiNO₃ 137.90 g./liter
or Li₂CO₃ (/ HNO₃ to dissolve) 73.89 g./liter
2. Dilute Li solution: 20 ml. of stock diluted to 1 liter with distilled water
3. Stock mixed sodium and potassium standard
KCl (dry) 0.4475 g. ;
NaCl (dry) 9.3526 g.) Diluted to 1 liter with distilled water
This makes a solution 160 meq. Na and 6 meq. K per liter
4. Dilute mixed standard - see under Procedure

Procedure: (using the Baird flame photometer)

1. Preparation of serum:

Collection should be made in plain tubes, avoiding rough handling and hemolysis which will release potassium into the serum. Merely allowing serum to stand over cells for prolonged periods will cause high serum K values.

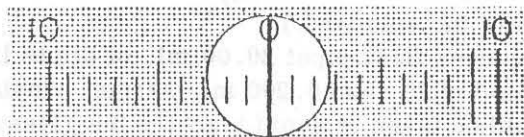
2. Lighting and adjusting burner:

- a. Turn on air at stopcock,
- b. Turn air regulator control clockwise until 5 lbs./sq. inch is registered on the dial.
- c. Turn on gas and light burner at top with lighted match.
- d. Increase air pressure to 10 lbs./sq. inch.
- e. Allow apparatus to warm up 15-20 minutes before calibration.

Sodium Standardization

1. Preliminary adjustment

- a. Rotate the filter wheel to the Na position. The filter wheel must **CLICK** into position.
- b. Set the SENSITIVITY knob to the indicated mark (about 10% of full rotation).
- c. Set the BALANCE control at 700.
- d. Set the selector SWITCH at DIRECT.
- e. Using the galvanometer knob, adjust the galvanometer to read zero at the center of the top scale, thus:



2. Standardization

- a. Set the selector SWITCH to **INTERNAL STANDARD**
- b. Pour dilute mixed standard into the funnel.
- c. Adjust the SENSITIVITY to return the galvanometer to zero. (After this point, **DO NOT READJUST THE SENSITIVITY!**)
- d. After the funnel has run dry, set SWITCH to **DIRECT**.

- e. Using the galvanometer knob, adjust the galvanometer to read zero.
- f. Reset the SWITCH to INTERNAL STANDARD. Pour more dilute mixed standard into the funnel and adjust the BALANCE control to zero the galvanometer.
- g. Repeat steps d, e, and f until it is not necessary to make any adjustment to zero the galvanometer when
 - (1) the funnel is dry and the SWITCH at DIRECT
 - (2) the funnel is full of dilute mixed standard and the SWITCH at INTERNAL STANDARD.

Potassium Standardization:

1. Preliminary adjustment

- a. Rotate the filter wheel to the K position.
- b. Set the BALANCE control at 250.
- c. Set the SENSITIVITY knob at the indicated mark (about 50% of full rotation).
- d. Set the selector SWITCH at DIRECT.
- e. Using the galvanometer knob, adjust the galvanometer to read zero at the center of the scale.

2. Standardization:

Carry out steps a-g as under sodium standardization.

Procedure for 1.00 ml. serum:

1. Prepare dilute mixed standard by rinsing out a glass-stoppered 100 ml. volumetric flask with dilute lithium solution at least three times
2. Pipet 1.00 ml. of stock mixed standard into the flask.
3. Dilute to the mark with dilute lithium solution and mix well by repeated inversion with shaking.
4. Prepare the serum dilution in a similar manner using 1.00 ml. serum.
5. Compare the serum dilution with the dilute standard as outlined below.

Procedure for 0.200 ml. serum:

1. Prepare dilute mixed standard by rinsing out a glass-stoppered 100-ml. volumetric flask with dilute lithium solution at least 3 times.
2. Fill to the mark with dilute lithium solution.
3. Add to this (100 ml. of dilute lithium solution), 1.00 ml. of stock mixed standard. (This results in a 1:101 dilution of the standard.)
4. Into a dry 50-ml. Erlenmeyer flask pipet 20.00 ml. of dilute lithium solution. With a "to contain" 0.20 ml. blood pipet, add 0.200 ml. serum, rinsing back and forth at least eight times. Care should be taken to avoid touching the glassware with the fingers since perspiration is very high in Na and K. This results in a 1:101 dilution of serum
5. Compare the serum dilution with the standard as outlined below.

Comparison of Standard and Unknowns:

1. Standardize the instrument for sodium or for potassium as outlined above.
2. Pour dilute mixed standard into the funnel and adjust the **BALANCE** control to zero the galvanometer. Record the dial reading R_s .
3. After the standard has passed through the aspirator, pour some of the serum dilution into the funnel, and again adjust the **BALANCE** control to zero the galvanometer. Record the reading R_u .

Calculation:

$$(R_u/R_s) \times C_s = C_u$$

Example:

<u>Na</u>	<u>K</u>
$R_s = 710$	$R_s = 256$
$R_u = 606$	$R_u = 207$

The standard contained 160 meq Na and 6 meq K per liter.

Na $(606/710) \times 160 = 137$ meq Na/liter.

K $(207/256) \times 6 = 4.85$ meq K/liter

Interpretation:

See under Electrolytes (Appendix p. 321)

Note: Exact amounts of lithium to use and exact setting of the **BALANCE** control will vary with the sensitivity of the photocells.

SULFA DRUGS

Blood, Urine and Spinal Fluid

Reference:

Bratton, A. C. and Marshall, E. K.: J. Biol. Chem. **128**, 537-550 (1939).

Principle:

The drug is diazotized and, after excess nitrous acid has been destroyed by sulfamic acid ($\text{HNO}_2 + \text{HOSO}_2\text{NH}_2 = \text{H}_2\text{SO}_4 + \text{H}_2\text{O} + \text{N}_2$), is coupled with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a colored product (an azo dye).

Reagents:

Saponin, 0.05%. 0.5 g. in 1000 ml. of water.

Trichloroacetic acid (TCA), 15% in water and 3% in water. The U.S.P. grade is satisfactory.

Sodium nitrite, 0.1% in water. Make fresh from 50% NaNO_2 (refrig.).

Ammonium sulfamate ($\text{NH}_4\text{OSO}_2\text{NH}_2$), 0.5% in water. Make fresh.

Coupling reagent. N-(1-naphthyl)-ethylenediamine dihydrochloride, 0.1% in H_2O . Fresh daily.

Stock standard sulfanilamide. Weigh accurately 0.1000 g. of sulfanilamide, transfer quantitatively to a one-liter volumetric flask, dissolve in hot water, cool to room temperature, dilute to volume, and mix. 1 ml. = 0.1 mg. This solution will keep several months in a refrigerator.

Dilute standard. Into a 100 ml. volumetric flask containing 18 ml. of 15% TCA pipet 3 ml. of stock standard, dilute to volume, and mix. Ten ml. of the dilute standard contains 0.03 mg.

Procedure:

Into a 50 ml. volumetric flask place 15 ml. of 0.05% saponin solution. Add 1 ml. of blood. When laking is complete, add 10 ml. of 15% TCA. Dilute to the mark. Mix. Filter. Transfer 10 ml. of filtrate, 10 ml. of the dilute standard, and 10 ml. of 3% TCA respectively to three dry photometer tubes, and treat each as follows: Add 1 ml. of 0.1% NaNO_2 solution. Mix. Let stand 3 min. Add 1 ml. of 0.5% ammonium sulfamate. Mix. Let stand 2 min. Add 1 ml. of coupling reagent. Mix. Let stand 10 min. Read in photometer.

Photo- meter tube no.	TCA blood filtrate ml.	Dil. std. ml.	3% TCA ml.	0.1% NaNO_2 ml.	Mix, let stand 3 min. Add ammonium sulfa- mate, ml.	Mix, let stand 3 min. Add coupling reag., ml.	Mix, let stand 10 min. Read in Photometer T D
1	10	-	-	1	1	1	- -
2	-	10	-	1	1	1	- -
3	-	-	10	1	1	1	<u>100.0</u> <u>0.000</u>

Calculation:

$$(D_u/D_g) \cdot 0.03(100/0.2) = (D_u/D_g) \cdot 15 \text{ mg. of drug/100 ml. blood}$$

Comments:

Saponin can be replaced by water if enough time is allowed for laking to become complete before addition of CCl_3COOH . Some of the drug is present in an acetylated form. Determination of this form requires hydrolysis of the filtrate with acid before diazotization and coupling. Some sulfa derivatives are so little soluble that it is advisable to add 5 ml. of 2.5 N NaOH to the water in which the drug is dissolved to make the stock standard. Amount of color developed by equal weights of sulfa drugs is approximately inversely as their molecular weight. It is better to use as a standard the particular derivative which is being determined.

For urine, use a 1 to 10 dilution with water. Saponin is not needed. Use the procedure as for blood on this dilution including the treatment with TCA.

THIOCYANATE Serum

Reference:

Bowler, R. G., *Biochem. J.* **38**, 385 (1944).

Principle:

A protein-free filtrate (trichloroacetic acid) is reacted with an excess of ferric ions in 0.5 N HNO_3 - which have been found to be optimum. The red color (ferric thiocyanate) is unstable especially in sunlight. The color should be read immediately using 515 m μ .

Reagents:

1. Trichloroacetic acid, 10% solution. Dissolve 10 grams in water, make to 100 ml. and filter.
2. Ferric nitrate-nitric acid reagent. Dissolve 80 grams of ferric nitrate $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$, in 250 ml. of 2 N nitric acid, make to 500 ml. with water and filter. Concentrated nitric acid is approximately 16 N so that 2 N acid contains very nearly 31.5 ml. of concentrated acid in 250 ml. Keep in the dark.
3. Standard thiocyanate solution. Prepare a standard solution containing 10 mg. SCN^- per 100 ml. of solution, by diluting 1 to 100 with water a thiocyanate solution containing 1 gram SCN^- per 100 ml. Standardize against silver nitrate solution. Twenty ml. of N/10 silver nitrate would require 11.6 ml. of the thiocyanate solution. Or, if N/10 thiocyanate is kept in the laboratory, the standard thiocyanate (10 mg. per 100 ml.) can be prepared by diluting 1 ml. of this to 58 ml. with water.

Procedure:

Pipet 1.0 ml. of serum into a test tube, add 6.5 ml. H_2O and 5.0 ml. 10% CCl_3COOH . Mix and allow to stand 10 - 15 minutes. Filter through Whatman filter paper #40.

To 5.0 ml. of filtrate add 5.0 ml. ferric nitrate reagent and mix well. (Keep out of bright light).

Prepare a blank, using serum known not to contain thiocyanate and a standard as follows: 1 ml. normal serum + 5.5 ml. H_2O + 1.0 ml. thiocyanate standard and 5.0 ml. 10% CCl_3COOH .

Calculation:

$$(\text{D}_u/\text{D}_s) \times 10 = \text{mg. SCN}^-/100 \text{ ml. serum.}$$

Interpretation:

Thiocyanates are used in the treatment of hypertension and in the estimation of total body water. Above about 12 mg. % toxic symptoms may appear. Therapeutic levels are about 10 mg. %.

THYMOL TURBIDITY

Reference:

Huerga, J. de la, and Popper, H., J. Lab. Clin. Med., **34**, 877 (1949).

Rucci, H., J. Lab. Clin. Med., **32**, 1266 (1947).

Principle:

It has been found that sera added to thymol solutions give turbidities, especially in liver disease such as infectious hepatitis. The amount of turbidity is measured against the standard turbidity tubes described under TURBIDITY STANDARDS, p. 329.

Solutions:

1. Thymol in alcohol 10% - 10 g. anhydrous thymol dissolved up to 100 ml. with 95% ethyl alcohol. Keep in refrigerator.

2. Half-strength Thymol-Buffer. Use only until turbid.

Barbital (Barbituric acid) 2.76 g.

Sodium Barbital 2.06 g.

Make up to 800 ml. in a 1-liter volumetric flask, add 5.0 ml. of 10% alcoholic thymol solution, shake well to dissolve and fill with water to the mark. Mix.

3. Full strength Thymol-Buffer (use only until turbid). 0.5 ml. of 10% thymol is diluted to 100 ml. with half-strength Thymol-Buffer, shaking well to dissolve.

Procedure:

To 6.0 ml. of full strength Thymol-Buffer in a large (22 x 200 mm.) standardized photometer tube, add 0.100 ml. of serum, using a "to contain" pipet. Mix, allow to stand 30 minutes and read against Thymol-Buffer blank, at 644 mu.

Calculation:

From transmission reading, look up optical density and refer to graph of optical density vs. thymol or turbidity units.

Example:

A serum gave a T of 83.6 corresponding to an optical density of 0.078. This represents 1.8 turbidity units as read from the graph. Normally, 0-4 units are obtained, (usually 1-2). Elevations up to 20 units are seen in liver disease, especially infectious hepatitis and cirrhosis.

Precautions:

1. Clear, uncolored thymol buffer is essential.
2. Good mixing of serum and buffer is necessary.

Notes and Interpretation:

The change in and increase of gamma globulins are mainly responsible for positive results. Also, however, the amount of phospholipid, and qualitative changes in the albumin, are important.

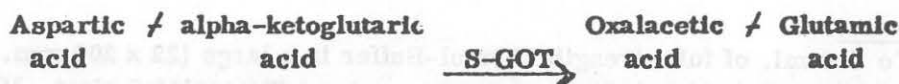
TRANSAMINASE - Serum (Serum glutamic-oxalacetic transaminase ("S-GOT"))

References:

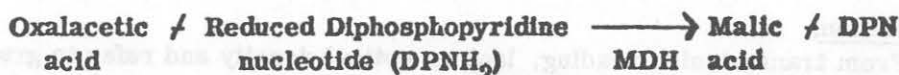
1. Steinberg, D., Baldwin, D., and Ostrow, B. H.: A clinical method for the assay of serum glutamic-oxalacetic transaminase. *J. of Lab. and Clin. Med.* **48**, 144-151 (1956).
2. Cabaud, P., Leeper, R., and Wroblewski, F.: Colorimetric measurement of serum glutamic oxalacetic transaminase. *Am. J. of Clin. Path.* **26**:1101-1105 (1956).
3. Wroblewski, F., Caband, P.: Colorimetric measurement of serum glutamic-pyruvic transaminase, *Am. J. Clin. Path.*, **27**, 235-9 (1957).

Principle:

Glutamic-oxalacetic transaminase catalyzes the transfer of an amino group from aspartic acid to alpha-ketoglutaric acid, yielding oxalacetic acid and glutamic acid, or the reverse:



This reaction is coupled with the malic dehydrogenase (MDH) reaction:



As fast as oxalacetic acid is formed it is reduced to malic acid by the action of MDH, provided the concentrations of both MDH and DPNH₂ are high enough. Under the specified conditions of substrate concentration, pH and temperature, the rate of disappearance of DPNH₂ is directly proportional to the S-GOT concentration. The reaction is followed by observing the decrease in the characteristic light absorption peak of DPNH₂ at 340 mu where DPN has virtually no absorption.

This test is highly specific for transaminase and is reproducible with a standard deviation of ± 6 per cent.

Apparatus:

This reaction can be followed in the Beckman DU spectrophotometer or the Bausch and Lomb's spectronic 20 colorimeter (specially calibrated at 340 mμ). Any spectrophotometer calibrated at 340 mμ can be used. The Beckman DU spectrophotometer, however, affords somewhat greater precision.

Reagents:

1. Potassium phosphate buffer, 0.1 M, pH 7.4.

13.97 grams buffer quality anhydrous K_2HPO_4 / 2.69 grams buffer quality anhydrous KH_2PO_4 made up to 1 liter with water. Store in the cold.

2. 0.2 M L-aspartic acid, sodium salt.

2.662 grams L-aspartic acid dissolved in 70 ml. of solution (1). Adjust to pH 7.4 (approximately 20 ml. 1 N NaOH). Dilute to 100 ml. Keep frozen.

3. Reduced diphosphopyridine nucleotide ($DPNH_2$).

Available in different degrees of purity. If using 70% pure $DPNH_2$, take up 10 mg. in 10 ml. of (1). If using 90% pure $DPNH_2$, take up 7.5 mg. in 10 ml. of (1). When diluted 1:10 as in the assay procedure this solution should give an optical density about 0.5 at 340 mμ.

4. Malic dehydrogenase (MDH).

Potency varies from batch to batch. Enzyme should be diluted with (1) so as to contain 2000 MDH units/ml. Refrigerate.

5. 0.1 M alpha-ketoglutaric acid, sodium salt.

1.47 g. alpha-ketoglutaric acid dissolved in 70 ml. of solution (1). Adjust to pH 7.4 (approximately 20 ml. 1 N NaOH). Dilute to 100 ml. Keep frozen.

Procedure:

1. For each serum to be assayed prepare two tubes as follows:

	<u>Control Tube 1</u>	<u>Assay Tube 2</u>
0.2 M aspartate	0.5 ml.	0.5 ml.
MDH (2000 units/ml.)	0.1 ml.	0.1 ml.
$DPNH_2$ (0.75 to 1.0 mg./ml)	---	0.3 ml.
Serum	0.3 ml.	0.3 ml.
0.1 M phosphate buffer	1.9 ml.	1.6 ml.

2. After thoroughly mixing, allow tubes to stand for 30 minutes. This pre-incubation period is crucial to exhaust the intrinsic $DPNH_2$ oxidizing capacity of the serum sample.

3. After 30 minutes add 0.2 ml. of 0.1 M alpha-ketoglutarate to each tube and mix again.

4. Using tube 1 as a blank, read the optical density of tube 2 at 340 mu exactly 2, 4, 6, 10 and 15 minutes after step 3. For samples of low activity another reading can be taken at 20 minutes. With samples of high activity the optical density may fall below 0.200 in the first 6 minutes, indicating that the GOT level is probably over 100 units/ml. To obtain an accurate value the test should be repeated using a 1:2 or 1:4 dilution of the serum (diluted with solution 1).

TEMPERATURE CONVERSION FACTORS

Temperature °C	f_T	Temperature °C	f_T
21	0.73	30	1.37
22	0.80	31	1.44
23	0.88	32	1.52
24	0.93	33	1.61
25	1.00	34	1.70
26	1.08	35	1.80
27	1.14	36	1.89
28	1.22	37	1.98
29	1.30	38	2.10

Calculations:

One unit of GOT is the amount of enzyme that will cause the reading at 340 mu to decrease at the rate of 0.001 O.D. units per minute per centimeter light path at 25°C. under the described conditions.

Before any assay results are accepted for calculation, the linearity of the reaction rate is confirmed by inspection or plotting of the readings. Within the limits of error for reading the instrument, the rate of change in O.D. should remain constant during the assay. If the rate is linear, the difference between any two readings can be used in calculating results. Errors due to instrument instability, reading errors, etc., are minimized by using the first and last readings.

$$\text{GOT (units/ml.)} = \left(\frac{\text{O.D.}_1 - \text{O.D.}_2}{t_2 - t_1} - \text{MDH blank*} \right) \times \frac{1}{f_T} \times \frac{1000}{\text{ml. serum}}$$

where:

O.D. ₁ = optical density at t₁

O.D. ₂ = optical density at t₂

t = time (minutes)

f_T = temperature conversion factor

*MDH blank = see the Notes Section for significance.

Sample Calculation: (A similar data sheet should be made out for all determinations).

Time (Min)	Optical density readings				
	2	4	6	10	15
Sample No. 1	0.490	0.482	0.474	0.457	0.437
Sample No. 2	0.475	0.470	0.455	0.427	0.392
Temperature					28°C

"Sample No. 1"

$$\begin{aligned} \text{GOT (units/ml.)} &= \frac{0.490 - 0.437}{15 - 2} \times \frac{1}{1.22} \times \frac{1000}{0.3} \\ &= 11.1 \text{ units/ml.} \end{aligned}$$

In calculating Sample No. 2 the optical density at t₁ would be 0.470 since it was at this point that the reaction began to fall at a constant rate.

Standardization:

A reference standard of the GOT enzyme is not at present available. However, if the following two criteria are met it can be concluded that the assay is working properly.

1. The curve for DPNH₂ disappearance is strictly linear in all assays.
2. The rate of the reaction is directly proportional to GOT concentration. Graded

aliquots of the same serum sample are assayed on the same day. The rates of DPNH_2 disappearance should be in direct proportion to the size of serum aliquot used.

NOTES

MDH Blank. Preparations of malic dehydrogenase generally contain small but measurable amounts of GOT activity. The level of this GOT activity should be checked for each batch of MDH. The assay is run in the usual manner, substituting phosphate buffer for serum, and the observed rate of fall in optical density at 340 m μ is subtracted from the observed rates in all assays. If the MDH blank is less than 0.0005 per minute it can be neglected for most purposes.

Temperature. As with all enzymatic reactions the rate of this coupled reaction varies markedly with temperature. For maximum accuracy and reproducibility, tubes should be incubated in a constant temperature bath. However, provided room temperature is sufficiently constant, it is possible to obtain accurate and reproducible results incubating the tubes in room air.

Normal red blood cells contain GOT at a much higher concentration than normal serum. Hemolysis must be avoided during collection of GOT concentrations in serum and plasma. Heparin and oxalate do not interfere with the assay. GOT in serum is stable at refrigerator temperatures for 3 weeks.

Glutamic-oxalacetic transaminase is only one of many transaminases. Other transaminases can be determined by this method by using the appropriate substrate and enzyme. For example glutamic-pyruvic transaminase can be determined by this procedure by substituting alanine for aspartic acid and lactic dehydrogenase for malic dehydrogenase.

When using a Beckman DU spectrophotometer it is not necessary to run a separate blank for each serum to be assayed. It is possible to assay three samples at a time by using a common blank (this common blank can be prepared by diluting serum with phosphate buffer in the ratio that it is diluted in the assay samples). Satisfactory results can be obtained by this modification.

It has been reported that aspirin administration can influence the serum activity of glutamic-oxalacetic and glutamic-pyruvic transaminases in children (Manso, C., A. Toranta, and I. Nydic. Effect of aspirin administration on serum glutamic-oxalacetic and glutamic-pyruvic transaminases in children. Proc. Soc. Exp. Biol. & Med. 93: 84-88 (1956).

Interpretation:

Normal values are usually between 10 and 33 units/ml. of serum for glutamic-oxalacetic transaminase. GOT levels rise within 24 hours and fall to normal by the

6th day after acute myocardial infarction. In recent myocardial infarction the values are elevated often to 100-200 units/ml. and occasionally to several thousand units. Elevations are also seen in jaundiced patients with active liver disease.

The measurement of serum glutamic-pyruvic transaminase alterations has been found to be a useful tool in the diagnosis and study of acute hepatic disease and appears to be more sensitive than S-GOT in depicting acute hepatocellular damage. Normal values for glutamic-pyruvic transaminase are usually between 5 and 35 units/ml. of serum.



A protein free filtrate is then prepared. A portion is assayed, and is compared photometrically with a standard curve solution treated similarly, using 515 mμ wave-length.

Reagents

1. Ethylenediaminetetraacetic acid (EDTA). (Trade names: Versene, Bacterisene) 1% pH 8.2. Weigh out 1.0 gram ethylenediaminetetraacetic acid, suspend in about 50 ml. of distilled H₂O, neutralize with NaOH to pH 8.2, transfer to paper or glass container and dilute up to 100 ml.
2. Urease solution. 0.5% Weigh out 0.50 g. of urease (crystalline, Sigma) and add to 100 ml. of the neutralized EDTA. Mix to dissolve. Keep refrigerated. This solution maintains its original activity for at least 4-6 weeks in the cold. Each bean meal extract may be used. See notes below.
3. Acetate buffer. Dissolve 100 g. $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 2\text{H}_2\text{O}$ and 1.7 ml. glacial acetic acid in water, dilute to 500 ml. and mix.
4. Urease-buffer mixture. Add 5 ml. urease solution and 5 ml. acetate buffer to 5 ml. H₂O and water. Vortex as may be used.
5. Nitro catalyst. 1% (w/v) NaNO_2 . Dissolve 1.0 g. NaNO_2 in 100 ml. distilled water.
6. Nitrophenol stock solution. Dissolve 0.400 g. of pure, dry nitro in water and dilute to 1 liter with water. Add 2 drops toluene. This solution contains 50 mg. of nitro nitrogen per 100 ml.
7. Nitro standard. Dilute 0.005 ml. of stock standard nitro solution to 100 ml. with water. Mix. Five ml. contains 0.02 mg. nitro nitrogen.
8. Ammonium molybdate, stock solution. Weigh out 0.5000 gram ammonium molybdate and dry, dissolve in water, and add one drop of concentrated H₂SO₄. Dilute to 1 liter with water. Five ml. contains 50 mg. ammonium molybdate per 100 ml.

UREA NITROGEN

Blood and Urine

Reference:

Boutwell, J.H., Jr.; Clin. Chem., 3, 205 (1957).

Principle:

A dilution of blood (or urine) in water is incubated with urease to convert urea to ammonia.



A protein free filtrate is then prepared. A portion is nesslerized, and is compared photometrically with a standard urea solution treated similarly, using 515 mu wavelength.

Reagents:

1. Ethylenediaminetetraacetic acid (EDTA). (Trade names, Versene, Sequestrene), 1%, pH 6.8. Weigh out 1.0 g. of ethylenediaminetetraacetic acid, suspend in about 50 ml. of distilled H_2O , neutralize with NaOH to pH 6.8 (nitrazine paper or glass electrode) and dilute up to 100 ml.
2. Urease solution, 0.5%. Weigh out 0.50 g. of crude commercial urease (Sigma #2) and add to 100 ml. of the neutralized EDTA. Mix to dissolve. Keep refrigerated. This solution maintains its original activity for at least 4-6 weeks in the cold. Jack bean meal extract may be used. See notes below.
3. Acetate buffer. Dissolve 100 g. $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ and 1.1 ml. glacial acetic acid in water, dilute to 500 ml. and mix.
4. Urease-buffer mixture. Add 2 ml. urease solution and 2 ml. acetate buffer to 6 ml. H_2O and mix. Prepare on day of use.
5. Zinc sulfate, 1%; $\text{Ba}(\text{OH})_2$, 0.06 N. See p. 76, Deproteinizing Methods.
6. Standard urea solution, stock. Dissolve 0.4287 g. of pure, dry urea in water and dilute to 1 liter; mix. Add 2 drops toluene. This solution contains 20 mg. of urea nitrogen per 100 ml.
7. Urea standard, dilute. Dilute 5.000 ml. of stock standard urea solution to 100 ml. with water. Mix. Five ml. contains 0.05 mg. urea nitrogen.
8. Ammonium sulfate, stock standard. Weigh out 0.9434 ammonium sulfate (pure and dry), dissolve in water, and add one drop of concentrated H_2SO_4 , dilute to 1 liter with water. Mix. Five ml. contain 20 mg. ammonia nitrogen per 100 ml.

9. Ammonium sulfate, dilute standard. Dilute 5 ml. of stock standard ammonium sulfate to 100 ml. with water. mix. Five ml. contains 0.05 mg. of nitrogen.

10. Nessler's Stock Solution (Koch-McMeekin). Into a 500 ml. Florence flask place 22.5 g. of iodine, 30 g. of KI, 20 ml. of water, and 30 g. of pure metallic mercury. Shake the mixture by rotation. The iodine and iodide will quickly dissolve, and the flask and contents will become hot. In a short while the red iodine color will begin to fade. Before all red color disappears cool rapidly under cold water, and when the solution has only a small amount of red color, decant the aqueous solution carefully from the excess mercury, which should be saved and recovered. Transfer all of the aqueous portion to a cylinder, and dilute up to 200 ml. with water.

11. Iodinated Nessler's Solution. To 100 ml. of Nessler's Stock Solution add 1.0 g. of pure I_2 (solid). Mix. Allow to stand until dissolved, mixing occasionally. This solution will require several days. If necessary, the solution may be used before the iodine is completely dissolved.

12. NaOH, 2.5 N. Dilute 139 ml. of 18 N NaOH (see Solutions p. 30) up to 1 liter with water. Standardize and adjust normality to a figure between 2.48 N and 2.52 N.

Procedure:

1. To 5.00 ml. distilled water in a test tube, add (by 8 times rinsing), 0.200 ml. blood from a pipet calibrated "to contain." Mix.

2. In addition to the blood tube, prepare similar tubes containing:

- 5 ml. H_2O
- 5 ml. dilute urea standard
- 5 ml. dilute ammonium sulfate standard (if desired)
- 1 - 5 ml. of a 1:200 dilution of urine (the exact amount depends on the urine urea concentration) made up to 5 ml. with water.

3. To each tube add 1.0 ml. urease buffer mixture. Mix. Incubate in water bath at $50^{\circ}C$. for 10 minutes.

4. Add to each tube 2.00 ml. of zinc sulfate, 1%. Mix.

5. Immerse tubes in a boiling water bath for 5 minutes. Remove and cool.

6. Add 2.0 ml. $Ba(OH)_2$, 0.06 N. Mix by vigorous shaking. Centrifuge.

7. Transfer 5 ml. aliquots (by means of cotton tipped pipet) to matched photometer tubes.

8. To each tube add 1.0 ml. Iodinated Nessler's Solution. Mix. Then add rapidly (blow in) 1.0 ml. 2.5 N NaOH, mix each tube immediately after alkalizing.

9. Allow to stand 5 minutes, read within an hour against the reagent blank set at 100.

10. If the transmittance is above 90 or below 10, repeat, with more or less sample or filtrate.

Calculations:

A. Blood. $C_u = (D_u/D_s) \times C_s \times \frac{V_{ref}}{V_{anal}}$; or $C_u = (D_u/D_s) \times 5(0.05/10) \frac{100}{5 \times 0.2/10.2}$

or $C_u = (D_u/D_s) \times 25.5 = \text{mg. urea nitrogen/100 ml.}$

B. Urine. Using 2 ml. of 1:200 dilution,

$$C_u = (D_u/D_s) \times 5 \times (0.05/10) \cdot \frac{V_{total}}{(5/10)(2/200)} = (D_u/D_s) \cdot 5 \cdot V_{total} = \text{mg. urea nitrogen/volume}$$

Comments:

1. NaOH, 0.06 N, may be used instead of Ba(OH)_2 , with only minute and occasional appearance of turbidities in the final solution.

2. Addition of iodinated Nessler's solution and NaOH 2.5 N should be carried out at room temperature.

3. Urease is protected from heavy metal poisons by EDTA, by blood proteins and by some other substances, but in urine dilutions and in pure urea solutions, not enough of this protection is present. Hence, care should be taken to separate tubes for Nesslerization (mercury containing) from those used for incubation. Urease poisoning accounts for the occasional apparent failure of urease to act.

4. The use of iodinated Nessler's solution prevents the reaction of such reducing substances as creatinine and uric acid with the alkaline Nessler's reagent. Other oxidizing agents have a similar effect.

5. The heating of the solutions in the presence of ZnSO_4 is necessary to insure complete removal of protein by the ZnSO_4 - Ba(OH)_2 reagents.

6. Reproducibility of color depends on rapid mixing when the alkali is added (Step 8).

7. Obviously, the sum of urine urea nitrogen and urine ammonia is determined by this procedure. If a correction for urine ammonia is desired, add the ZnSO_4 before the urease, heat in the boiling water bath immediately, cool and proceed as above, using the value obtained as a blank value to be subtracted from the urea value.

8. If greater photometric accuracy in the low normal range is desired, use 5.0 ml. of a Folin-Wu 1:10 blood filtrate (instead of the 5.0 ml. of water / 0.2 ml. blood). This then will result in a 0.25 ml. aliquot and $C_u = (D_u/D_s) \cdot 10$ mg. % urea N in blood.

9. Urine Ammonia - Proceed as above with the following changes:

- a. Use a 1:100 dilution of urine.
- b. Omit the addition of urease - buffer. Step 3.
- c. Omit the urea standard; use the ammonium sulfate standard.

Calculation: Using 5.00 ml. of 1:100 dilution

$$C_u = (D_u/D_s) \cdot 100 = \text{mg. NH}_3 \cdot \text{N per 100 ml. urine}$$

10. Jack bean meal extract may be prepared as follows:

Urease extract. Weigh out 1.0 g. of ethylenediaminetetraacetic acid (EDTA, Versene, Sequestrene), suspend it in about 50 ml. of distilled water, and add 10% NaOH with stirring until the EDTA goes into solution and a pH of 6.8 is reached. In this solution dissolve 20 g. of $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ and 0.22 ml. of glacial acetic acid, dilute to 100 ml. with water, and mix. Shake this solution with 15 g. of jack bean meal for 15 minutes. Centrifuge at 2000 rpm for 2 minutes, and decant all liquid portion that will pour off. Addition of toluene and refrigeration will preserve the extract for several weeks. Mix before using. This extract has a pH around 6.25 at room temperature.

Acetate buffer solution. Suspend 10 g. of EDTA in about 500 ml. of water, dissolve with NaOH and bring to pH 6.8. Add 50 g. of $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ and 0.55 ml. of glacial acetic acid, dissolve, dilute to 1 liter, and mix.

Urease-buffer mixture. Add 1 ml. of urease extract to 50 ml. of acetate buffer solution. Stir up before use. Make fresh daily.

UREA

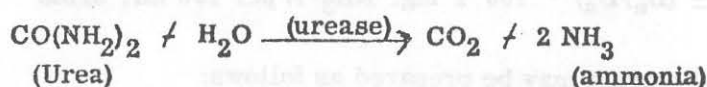
Blood and Urine

References:

1. Karr, W. G.: J. Lab. and Clin. Med. 9, 329 (1924).
2. Connerty, H. V., Briggs, A. R. and Eaton, E. H., Jr., Am. J. Clin. Path., 25, 1321 (1955).

Principle:

A tungstic acid filtrate is treated with urease (using a pyrophosphate buffer) to convert urea to ammonia. The ammonia is then reacted with Nessler's reagent to produce a colloidal yellow color which is compared to the color produced by a standard urea solution treated similarly.



Reagents:

1. Deproteinizing reagents, see p. 76.
2. Pyrophosphate buffer. Dissolve 14 g. sodium pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$) and 2.0 ml. phosphoric acid (H_3PO_4 , 85%) in distilled water and dilute to 100 ml.
3. Urease solution.

Into a 200 ml. flask, place 100 ml. of about 30% ethyl alcohol. Add 30 g. jack bean meal and 1 ml. pyrophosphate buffer. Stopper and shake vigorously for five minutes. Centrifuge for 1/2 hour in 12 ml. heavy wall centrifuge tubes, covering the mouth of the tube with aluminum foil. Transfer the supernatant to a glass-stoppered bottle and store under refrigeration.

4. Potassium mercuric iodide solution:

Into a 500-cc. Florence flask place 150 gm. of potassium iodide (KI) A.C.S., 100 gm. of elemental iodine, A.C.S., and 100 ml. of distilled water. Shake the contents of the flask until the iodine has completely dissolved. Add 150 gm. of metallic mercury, A.C.S. Shake the flask continuously and vigorously for 7 to 15 minutes until the dark red color of the iodine has become visibly lighter though still red. The solution becomes quite hot at this stage. After the solution becomes pale though still red, cool the flask under a running water tap and continue shaking until the reddish color of iodine has been replaced by the greenish-yellow color of the double iodide. If the solution is properly prepared, it is clear and there is a residue of shining metallic mercury. Decant the solution in a 2-liter volumetric flask, washing the mercury and flask with copious quantities of distilled water. Dilute the solution and washings to 2 liters. If the cooling was begun in time, the resulting reagent is clear enough for immediate dilution with 10 per cent sodium hydroxide and water for preparation of Nessler's solution. Never discard mercury by pouring it into a sink because it will alloy with the lead of plumbing fixtures and later cause leaks.

5. Nessler's solution

Into a 2-liter volumetric flask place 300 ml. of potassium mercuric iodide

solution and 1,400 ml. of 10 per cent sodium hydroxide. Dilute to the mark with distilled water and mix thoroughly. Allow the solution to stand several days before use to allow the yellow precipitate to settle.

6. Stock urea standard:

Dissolve 0.1286 gm. of urea, weighed on an analytical balance, in distilled water and dilute to 200 ml. in a volumetric flask.

7. Working urea standard:

Transfer 5 ml. of stock urea standard with a volumetric pipet to a 100-ml. flask and make up to the mark with distilled water. 1 ml. of urea standard contains 0.015 mg. of nitrogen.

8. I_2 -KI solution

Add 2 g. I_2 to 3 g. KI, dissolve in a little water and dilute to 100 ml.

Procedure:

1. Prepare a 1:10 tungstic acid filtrate of whole blood, see p. 76.
2. Into a clean, dry test tube calibrated at 9 ml., place 1 ml. of the protein-free filtrate.
3. Into similar test tubes, place 1 ml. of water (for a blank) and 1 ml. of urea standard containing 0.015 urea nitrogen.
4. To each tube add 1 drop of pyrophosphate buffer and 1 drop of urease solution. Mix well.
5. Incubate at 50°C ($\pm 5^{\circ}\text{C}$.) for 10 minutes. Cool. Add 1 drop of 2% I_2 -KI solution.
6. Dilute each tube to exactly 9.0 ml. Mix well.
7. Cool to below 20°C . in a water bath or refrigerator.
8. Add to each tube in turn 1.0 ml. alkaline Nessler's solution, mixing immediately after each addition.
9. Allow to stand for 5 minutes, read against the reagent blank within 30 minutes using a wavelength of 500 mu (or a green filter).

Calculation:

$$(\text{Du/Ds}) \times C_s \times \frac{100}{0.1} = \text{mg. urea N per 100 ml. blood}$$

$$(\text{Du/Ds}) \times 0.015 \times 1000 = \text{mg. urea N per 100 ml. blood}$$

or

$$(\text{Du/Ds}) \times 15 = \text{mg. \%urea N}$$

Urine Urea:

1. Dilute urine 1 to 500 with water.
2. Proceed as outlined above, Steps 2-9.

UREA Visual Colorimetric Determination

Principle:

Since the yellow Nessler's- ammonia complex color is difficult to measure by visual colorimetry, it is necessary to work with more concentrated solutions.

Procedure:

1. Into a 25 x 200 mm. test tube calibrated at 20 ml., place 5.0 ml. of a protein-free filtrate of whole blood.
2. Into similar test tubes place 5.0 ml. of a urea standard containing 0.075 mg. urea nitrogen.
3. Add 5 drops of pyrophosphate buffer and 5 drops of urease solution. Mix well.
4. Incubate at 50°C. (± 5°C.) for 10 minutes. Cool.
5. Add 2 drops 2% I₂·KI. Dilute to 20.0 ml. Mix well. Cool to below 20°C.
6. Add with immediate rapid mixing 2.0 ml. alkaline Nessler's solution.
7. Allow to stand 5 minutes and compare against the standard in a visual colorimeter. If possible a blue or green light source should be used.

Calculation

$$\frac{R_s}{R_u} \times C_s \frac{100}{0.5} = \text{mg. urea N per 100 ml. whole blood}$$

$$(R_s/R_u) \times 0.075 \frac{100}{0.5} =$$

$$(R_s/R_u) \times 15 = \text{mg. \% urea N}$$

Urine Urea:

Prepare a 1:500 dilution and proceed as above.

Interpretation:

Normal range: 8-18 mg. urea N per 100 ml. blood
(equivalent to 20-35 mg. urea)

Increased values are seen in renal failure, urinary obstruction, and oliguria. In acute nephritis, values as high as 200 mg. % may be reached. Decreased values are seen in acute hepatic failure and in normal pregnancy.

URIC ACID

Serum and Urine

Reference:

- Brown, H., J. Biol. Chem. 158, 601, (1945).
Buchanan, O. H., Christman, A. A. and Block, W. T., J. Biol. Chem. 157, 181, (1945).
Bermann, F., and Kikstein, J. Biol. Chem., 211, (1) 149. (1954).

Principle:

This method, like most, depends upon the great ease with which uric acid is oxidized in either acid or alkaline media. The oxidation is carried out by a special phosphotungstic acid in the presence of alkali and sodium cyanide (to intensify the color obtained).

Reagents:

1. Uric Acid Reagent (Phosphotungstic Acid): Into a 1000 ml. round bottom flask, which has been cleaned with dichromate sulfuric acid cleaning solution, place 150 ml. water, 100 g. $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$ (Folin), 16.3 ml. of 85% (13.8 M) H_3PO_4 and 16.8 ml. conc. H_2SO_4 (18 M.) Mix. Boil gently, two hours, using a reflux condenser or funnel-flask combination. Cool, and dilute to one liter with water. The reagent is stable indefinitely.
2. Sodium Cyanide - Sodium Carbonate. Dissolve 60 g. NaCN and 20 g. Na_2CO_3 (anhydrous) dilute to one liter with water. Keep refrigerated.
3. Urea - 50%. Dissolve 50 g. reagent grade urea up to 100 ml. with water.
4. Urea-Cyanide. Just before use mix equal volumes of reagents (2) and (3). Discard excess each day. Do not keep.
5. Uric Acid Stock Standard. Place 1.000 g. uric acid in a funnel on a 300 ml. Erlenmeyer flask. Place about 0.45 - 0.50 g. lithium carbonate in a beaker in about 150 ml. of water and heat at 60°C . and stir until all is dissolved. Rinse the uric acid into the flask with this hot carbonate solution and shake until the uric acid is dissolved, cool under running water and transfer to a one-liter volumetric flask. Rinse and dilute to a volume of 400-500 ml. Add 25 ml. of 40% formaldehyde and after thorough mixing add 3 ml. of glacial acetic acid and dilute to mark, Keep in a tightly stoppered bottle (brown) in the dark, 1 ml. = 1 mg.
6. Uric Acid Standard Dilute. Transfer 1.000 ml. of uric acid stock standard to 250 ml. in a volumetric flask, dilute to mark with water. Make fresh each day. 1 ml. 0.004 mg.

Procedure:

- A. Deproteinization: The usual 1:10 tungstic acid filtrate of serum (Protein-free Filtrates, p. 76 may be used).
- B. Color development: To a photometer cuvet add 1 ml. H_2O , 1 ml. serum filtrate and 4 ml. urea-cyanide mixture, mix well. Then add 1 ml. uric acid reagent, and immediately mix well.

Set up a blank tube (2 ml. H_2O); S_1 tube (1 ml. H_2O / 1 ml. dilute standard); and S_2 tube (2 ml. dilute standard) and develop color as above.

Allow all tubes to stand at room temperature for 60 minutes and compare photometrically (640 m μ).

Calculation:

$$(D_u/D_s) C_s \frac{100}{0.1} = C_u \text{ (mg. per 100 ml.)}$$

$$\text{For } S_1: (D_u/D_s) 0.004 \times \frac{100}{0.1} = (D_u/D_s) \times 4 = \text{mg. \% uric acid}$$

Urine:

A dilution of urine (1:100 usually is sufficient) is made and treated as blood filtrate.

Notes:

Sodium cyanide purity is essential to the success of the determination. Impure cyanide gives insufficient color, increases instability of the color, and results in the development of a yellow or brown color upon standing. Purification can be made as follows: (Medical and Public Health Laboratory Methods, Simmons, J. T., Gentzkow, C. J., Lea & Febiger, 1955). In a good hood, add 225 g. NaCN (reagent grade previously found to be unsatisfactory) to a 500 ml. flask. Add 200 ml. boiling water. Stir a few minutes. Filter through filter paper using vacuum. Cover. Allow to cool overnight in the refrigerator. Collect crystals using vacuum filter. Wash twice with 20 ml. ice water. Partially dry, store moist in a brown jar. Substances other than uric acid influence the amount of color obtained, so the method is not entirely specific. Also some of the uric acid is lost by deproteinization. Another difficulty, eliminated in the procedure given, was the fact that Beer's Law was not obeyed. In the present procedure Beer's Law is obeyed up to 0.01 mg. uric acid in the tube, and this is represented by an optical density of about 0.730, and corresponds to 10 mg. % uric acid.

**ZINC TURBIDITY (gamma globulin)
Serum**

Reference:

Kunkel, Henry G., *Proc. Soc. Exp. Biol. & Med.*, **66**, 217-224 (1947).

Principle:

The gamma globulin is precipitated by zinc sulfate at a pH of 7.5 (barbiturate buffer). The turbid solution is measured photometrically, at 644 mu, using the standard turbidities of Shank and Hoaglund (see *Turbidity Standards*, p. 329. but expressed in MacLagan units.

Reagents:

Mix the following reagents:

ZnSO ₄ ·7H ₂ O	0.024 g. (analytical balance)
Barbituric acid	0.280 g.
Sodium barbiturate	0.210 g.
H ₂ O	To make 100 ml.

Procedure:

1. Place 6.0 ml. of the zinc reagent in a photometer tube.
2. Add with a micro-pipet 0.100 ml. of serum, rinsing out 6-8 times.
3. Mix well. Allow to stand 30 minutes.
4. Shake. Read in photometer against a reagent blank, using 644 mu.

Calculation:

1. Look up optical density (T vs D table, Appendix)
2. Refer to calibration chart of turbidity for units of turbidity p. 329.

Interpretation:

The normal range lies between 2 and 8 units of turbidity. Certain sera with globulin levels above 6 g. % may show values as high as 80 units. The reaction is not specific for liver disease. The test was advocated by the author as being of particular value when applied serially throughout the course of acute illness. He found the zinc turbidity test to be the most sensitive method of detecting a lingering hepatitis. He also used it to follow development of antibodies after scarlet fever convalescence and in developing rheumatic fever.

TOXICOLOGY

From the history of ancient medicine, records and traditions indicate that even before the development of modern scientific methods there was in the field of medicine a knowledge of the action of venom poisons upon various animals. Although the medical profession of the early ancients consisted of superstitious beliefs, it was due to this very practice that there developed a school of anatomical observations. This was the observation of the entrails of animals. The action of poison was, therefore, observed.

Ancient literature, also, bears many allusions to the knowledge of poisons. Ovid, the poet, for example, between the years 43 B.C. and 17 A.D., wrote that the arrows of Hercules were charged with the venoms of the Lernean serpent. It is from this method of poisoning that the word poison (Latin - toxicum for poisoning, originally arrow poisoning) is derived (1).

Toxicology, therefore, is the science of poisons, their origin, their properties, their action on living tissue, the treatment to counteract their harmful effects, and the methods of their detection by chemical or other means (2).

In order to understand the true meaning of toxicology a definition is needed for a poison, which may be thought of as any substance which when introduced into the living body and acting chemically, is capable of causing serious disturbance of body functions or death of an ordinary individual with average health (3). For our purposes in the Navy, only the poisons which need be present in relatively small quantity in order to cause illness or death are of significance.

Poisons originate from animal, vegetable, and mineral sources as well as by synthetic means. The properties of poisons may be acidic, basic, or neutral; organic or inorganic.

The action of poisons on the system disturbs the normal functioning of a vital body process. The common symptoms may vary from vomiting, convulsions, coma, delirium, paralysis, dilation or constriction of the pupils, rapid or slow respiration, to miscellaneous signs such as blood changes, disturbed vision, urinary changes, abnormal coloring of the skin, and characteristic odors on the breath.

The minimum lethal dose of a poison is the smallest quantity of the substance which has proven fatal. It varies considerably with each individual poison, and each poison differs from itself according to the mode of administration. Also certain individuals have idiosyncrasies which account for further variations of the action on the body. There are many factors which contribute to this widespread action.

The form in which a solid, liquid, or gaseous material may be taken varies considerably; particle size and solubilities being instrumental in affecting toxicity. The poison may also enter the body in a variety of ways. The routes of administration in order of speed of absorption are intravenous, inhalation, intramuscular, rectal, vaginal, and oral. The oral route being last because of slow absorption from the stomach. Also poisons are often partially eliminated by vomiting, and protein foods in the stomach act in some cases as an antidote for metallic poisons.

The minimum lethal dose of a poison for an average 160 pound man varies from 10 mg. aconitine, 60 mg. nicotine, 100 mg. strychnine, 200 mg. arsenic, 1 to 6 gm. barbiturate, 15 gm. D.D.T., to one quart of whiskey (4).

The onset of illness is usually sudden, but not always. Symptoms usually but not necessarily follow the taking of food, drink, or medicine. The usual steps of treatment taken are:

- a. The removal of the poison from the body.
- b. The administration of antidotes.
- c. Supportive or symptomatic therapy.

In the removal of poison from the body there are two areas of consideration - one is the artificial expulsion and the other is natural elimination. Toxic substances can be removed by the use of the stomach tube, unless corrosives or strychnine are suspected. An emetic is very useful and easily obtainable. The chief avenues of natural elimination are via the kidneys, lungs, skin, and intestines.

In considering the second step in treatment, an antidote is any agent which will remove or prevent the absorption of a poison, or change its toxic properties, or counteract its physiologic effects. Antidotes act mechanically by removing the poison or by preventing its absorption by enveloping the poison in some insoluble material. They act chemically by changing the composition of the poison so that an insoluble compound is formed or by oxidizing the poison to nontoxic constituents. A physiologic antidote is one whose physiologic action on the body is opposite to that of the poison.

Symptomatic treatment, the third step, consists of cardiovascular, respiratory, or cortical stimulation and keeping the patient quiet and warm to combat collapse or shock.

The occurrence of poisoning may be accidental, suicidal, or criminal. Fortunately we do not encounter poisonings to any great extent of the latter type in the Navy. The rapid strides made in the science of toxicology have largely discouraged poisoning as a form of criminal attack. As suicidal agents, barbiturates and carbon monoxide are two that are often employed.

As a rule, the liver is the organ which detoxifies most of the poisons held in the system. The lungs are the principal organ of excretion of poisons producing their effects by inhalation.

The purpose of the Toxicology Laboratory at the Naval Medical School, National Naval Medical Center, is to detect qualitatively and quantitatively toxic substances in organic and inorganic material. The detection of poisons in tissue is an intricate procedure which demands specialized training. Occasionally a poison can be detected by gross examination of various parts of the body. For example the heavy metals are excreted in the saliva and produce characteristic effects on the mouth and lips. However, chemical tests are the primary means of detection.

References

1. McNally, W.D., Toxicology, Industrial Medicine, Chicago, p. 1, 1937.
2. Webster, W.W., Legal Medicine and Toxicology, W. B. Saunders Company, p. 318, 1930.
3. Goodman, L. and Gilman, A., The Pharmacological Basis of Therapeutics, The MacMillan Company, New York, p. 23, 1941.
4. Kaye, S., Handbook of Emergency Toxicology, Charles C. Thomas, pp. 1-24, 1954.
5. Bamford, F., Poisons, Third Edition, The Blakiston Co., Philadelphia, 1951.
6. Gradwohl, Legal Medicine, Mosby, p. 599, 1954.
7. Thienes, L. H., and Haley, T. J., Clinical Toxicology, Lea and Fabiger, 1949.

REINSCH TEST FOR ARSENIC, MERCURY, BISMUTH, SILVER,
AND ANTIMONY, USING MINCED TISSUE, BLOOD OR URINE

Reference:

J. F. Pract. Chem. Leipz. 24, 244 (1841)

Principle:

The common metal poisons combine with or form a deposit on copper in an acid media.

Apparatus:

No special apparatus needed.

Reagents:

1. 5 per cent HCl solution.
2. Arsenic-free copper foil strips (1/8" x 3/4") or 18 gage wire (coiled 1/4" x 1/2").

Procedure:

1. Place 5 - 10 g. of material in a suitable beaker or flask.
2. Add 25 to 35 ml. of 5% HCl solution.
3. Add one piece of Cu foil or wire which has been cleaned with concentrated HCl until bright and shiny. (Immerse strip in acid for 1-2 min.)
4. Cover flask or beaker with watchglass to decrease evaporation.
5. Place flask on hotplate and bring to boil.
6. Check from time to time to notice any change in color of the copper.
7. Add 5% HCl solution as needed to replenish loss due to evaporation.
8. Boil for 45 minutes.
9. Remove Cu, wash with water, alcohol, and ether, then examine.
10. Silvery gray indicates Hg, shiny gray Ag, metallic blue-black Bi, and black either As or Sb.
11. A reagent blank is run for comparison.

Calculations: None.

Standardization or Calibration: None.

Notes:

1. Do not handle Cu with fingers. Use forceps.

2. The HCl concentration in the test must be from 2 to 8 per cent. If less than 2 per cent certain arsenic compounds will not deposit. If more than 8 per cent the arsenic may be lost as arsine gas.

3. The deposit is not formed in the presence of oxidizing agents.

4. Compounds also causing stains on the Cu include sulfur (most common), selenium, gold, platinum, and palladium.

Interpretation:

1. This test is significant only when negative.
2. A positive test must be confirmed by other tests.
3. Sensitivity of tests as follows:

As	1 microgram/gm or ml		
Bi	5	"	"
Hg	25	"	"
Sb	5	"	"
Ag	10	"	"

ARSENIC DETERMINATION IN ORGANIC MATERIAL

References:

1. Association of Official Agricultural Chemists, 7th Ed., p. 369 (1950)
2. Sandell, E.B.: Ind. & Eng. Chem. (Anal. Ed.) 14, 82 (1942)
3. Bamford, F.: Poisons, The Plakiston Co., 3rd Ed., pp. 76-77 (1951)
4. Glaister, J.: Medical Jurisprudence and Toxicology, E. & S: Livingstone, Ltd., 9th Ed., p. 545-547 (1950)

Principle:

Organic material is destroyed by dry ashing. The arsenic (and Sb) are rendered nonvolatile by forming the stable magnesium pyroarsenate (and antimonate). The dry ash is dissolved in hydrochloric acid and introduced into a Gutzeit apparatus where the liberated arsine is collected on glass wool impregnated with mercuric bromide. If arsine is present, a yellow to dark brown stain will form which constitutes a qualitative test. If positive, the arsenic will then be subjected to a quantitative test by being converted to molybdenum blue and compared photometrically.

Apparatus:

1. Photoelectric colorimeter.
2. Gutzeit apparatus - the collection tube is packed in the lower end with absorbent cotton dampened with lead acetate solution. Blow gently thru tube to make sure gas can pass freely. The glass wool is inserted thru the upper-most end to about 1 1/2" from the bottom of the tube. It should not be tightly packed but should allow free passage of gases.

Reagents:

1. Stannous chloride solution - Dissolve 40 g. As-free $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in As-free conc. HCl and dilute to 100 ml. with same strength HCl.
2. Potassium Iodide solution - 15 g. As-free KI in water and dilute to 100 ml.
3. Lead Acetate solution - dissolve 10 g. of lead acetate $\text{Pb}(\text{Ac})_2 \cdot 3\text{H}_2\text{O}$ in water and dilute to 100 ml.
4. Zinc - 20-30 mesh - As-free.
5. Glass wool - pyrex brand. Wash in 10% NaOH and then in dilute HNO_3 (hot). Rinse with triple distilled water and dry. The wool is then impregnated by soaking in 5% mercuric bromide solution in 95% ethanol. Excess solution is pressed out between paper towels and allowed to air-dry.
6. 6 N sulfuric acid (approx.): Dilute 16.7 ml. concentrated H_2SO_4 acid to 100 ml. in distilled water.
7. KMnO_4 solution: Dissolve 0.1 g. of KMnO_4 in water and dilute to 100 ml.

Note: prepare fresh daily,

8. Ammonium molybdate solution - Dissolve 1 g. of ammonium molybdate in 10 ml. of water and 90 ml. of 6 N sulfuric acid.

9. Hydrazine sulfate solution - Dissolve 0.15 g. of hydrazine sulfate in 100 ml. of water. Note: prepare fresh daily.

10. COLOR REAGENT: Mix 5 ml. of ammonium molybdate solution and 5 ml. of hydrazine sulfate solution and dilute to 50 ml.

11. Standard Arsenic solution - Dissolve 1.32 g. of Bureau of Standards As_2O_3 in 25 ml. of 20% NaOH, saturate with CO_2 , and dilute to 1 liter with fresh triple distilled water. 1 ml. equals 1 milligram of arsenic. Prepare fresh daily a dilute standard by taking 1 ml. of the above standard and diluting to 1 liter with water. 1 ml. equals 1 microgram (μ).

12. Magnesium nitrate solution - Dissolve 200 g. of reagent magnesium nitrate in 160 ml. of water.

13. HCl - Approximately 22% As-free HCl.

14. MgO - Reagent grade powder.

Procedure:

1. Weigh 10-15 g. of organic material into a suitable Vycor or silica dish (not suitable for bone).

2. Add 4 ml. of $\text{Mg}(\text{NO}_3)_2$ solution for every 10 g. of tissue taken and sufficient MgO powder to render the mixture alkaline. (2g. MgO/10 g. tissue).

3. Evaporate to dryness on low hotplate by gradually increasing heat until dry, but avoid spattering. When dry, place in muffle furnace and slowly bring temperature up to 600 degrees C. Keep at this temperature at least 6 hours, preferably overnight. A fairly white colored ash should result. With organs high in blood, a yellow color due to iron will be present.

4. Cool, and add with stirring 22% HCl (caution, add slowly to avoid spattering). Use 10 ml. for every g. of MgO used. This will give a clear solution of the proper acidity for Gutzeit test. (If not clear, heat to boiling on hotplate, cool and filter).

5. Transfer to Gutzeit apparatus using 25 ml. of water. Add 2.0 ml. KI solution and 0.5 ml. SnCl_2 solution. Allow to stand for 20 minutes.

6. Add 3.5 g. of Zn and place the absorption and collection tube in place; allow the reaction to proceed for 90 minutes, keeping the apparatus immersed in a beaker of tap water.

7. A yellow to brown stain on the glass wool indicates the presence of arsenic. A blank control is run on all reagents used and a positive control of 5 micrograms of As. If the unknown is positive, a quantitative determination is made.

8. Prepare an oxidizing solution by mixing 8.0 ml. water, 1.6 ml. of 6 N H_2SO_4 , and 1.2 ml. of KMnO_4 solution.

9. 1.4 ml. of this mixture is placed in an Erlenmeyer flask (50 ml.) and the glass wool pledget is pushed out of the collection tube into it (using a small wooden applicator stick). A small amount of the solution is sucked up into the tube to wash any arsenic on the side of the tube into the main solution. Allow to stand 15 minutes.

10. Add 5 ml. of COLOR REAGENT and heat 15 minutes on steam bath. Cool and transfer to 25 ml. glass stoppered cylinder. Wash with small amounts of water. Add washings to solution and finally dilute to 10 ml. mark with water. Mix thoroughly.

11. Centrifuge and decant into a photometer cuvet. Determine % transmittance using 5 ml. of COLOR REAGENT diluted to 10 ml. as a blank. Read at 700 mμ if using a Coleman Jr. Spectrophotometer.

12. The arsenic content is determined by comparison with a standard curve obtained by treating known amounts of arsenic (5, 10, 15, 20, 25, 30, 35, 40 micrograms) with the above procedure. The blank is determined and, if appreciable, deducted from the amount calculated.

Notes:

1. Only triple distilled water (metal free) must be used for making up solutions.
2. All glassware must be washed with dilute nitric acid and rinsed with triple distilled water (metal free) until nitric acid is removed (usually 3 rinses is sufficient) before use.

Interpretation:

1. Arsenic is not a normal constituent of the human body. However, arsenic is so universally found that it may frequently be present in the human body in very minute amounts. When the greatest quantity detected is less than 1/10 milligram per 100 gm., but more than 1/50 milligram, the chemist must consult the medico-legal expert in charge of the case. Quantities in excess of those mentioned are reported as significant.

2. Arsenic is generally present in varying quantity in Egyptian soil.

3. The administration of arsenic on therapeutic grounds may be a cause for arsenic found in the body after death.

4. The smallest recorded fatal dose is 2 grams. Recovery has however, occurred after larger doses.

LIMIT TEST FOR MERCURY ON BIOLOGICAL MATERIAL

Reference:

Maren, T. H., J. Lab. & Clin. Med., 28, 1511 (1943)

Principle:

Both a qualitative and quantitative determination for mercury is carried out by forming a metal dithizonate color complex after destruction of biological materials.

Reagents:

1. Sulfuric acid, concentrated, ACS
2. Nitric acid, concentrated, ACS
3. Hydroxylamine HCl - 20% aqueous solution
4. 0.25 N HCl - 10.4 ml. analytical reagent diluted up to 500 ml. with triple distilled water. (If this does not produce satisfactory results use 45.0430 g. of constant boiling HCl diluted to 1 liter with triple distilled water).
5. Dithizone solution - 10 mg. per-liter - 1 ml. is equivalent to 2.5 micrograms of mercury. Make by diluting 10 mg. Eastman Diphenylthiocarbazone up to 1 liter with chloroform, ACS.
6. Standard mercury solution - dissolve 500 mg. metallic mercury in concentrated nitric acid and dilute to 500 ml. with triple distilled water. To 10 ml. of this solution add 10 ml. concentrated nitric acid and dilute to 1 liter with triple distilled water. 1 ml. equals 0.0100 mg. of mercury (10 micrograms per ml.).
7. Titration standard - dilute the above solution (1 ml. equals 0.0100 mg. of mercury) 1 to 10 with triple distilled water.

Procedure:

1. Weigh out 5 grams organic material into a 100 ml. long neck Kjeldahl flask.
2. Immerse flask in cold water and slowly add 20 ml. concentrated nitric acid.
3. After 10 minutes add 5 ml. concentrated sulfuric acid slowly; add 2 glass beads to prevent "bumping;" add a connecting bulb to the flask; apply heat gradually until full heat is used; more nitric acid must be added if charring occurs. Continue to heat until solution becomes clear.
4. Add 3 five ml. portions of triple distilled water, heating to SO_3 fumes after each addition.

Important: Cool flask before adding water each time and add the water slowly!!

5. Cool the clear mixture and add to the solution 75 ml. of 0.25 N HCl and 5 ml. hydroxylamine - HCl solution.

6. Heat to about 70° C. for 15 minutes; allow to cool (It may be cooled under the tap.)

7. Transfer to 500 ml. separatory funnel and dilute the solution to 250 ml. to bring pH to about 1.

8. Extract with successive 2 ml. portions of dithizone solution, shaking each 50 times using a glass-stoppered separatory funnel. Each pure orange extract will represent 5 micrograms of mercury. When a mixed color is obtained add standard mercury solution in 1 microgram portions (1.0 ml.) (shaking as above) until the orange endpoint is reached. Calculate as below.

9. A diluted standard control of 20 micrograms (20 ml.) is run through the procedure as a positive control.

10. A reagent blank is run with all determinations.

Notes:

1. All glassware must be washed with nitric acid and rinsed with triple distilled water until free of nitric acid (2 rinses is usually sufficient).

2. All reagents must be made up with triple distilled water when called for.

3. Platinum does not interfere at pH 1.

4. 10 micrograms of mercury can be detected in the presence of 500 micrograms of copper.

5. Gold gives a rose to purple color at this pH.

6. Silver interferes both mechanically and chemically by decolorizing dithizone solution.

Calculations: (Example)

1 ml. dithizone \times 2.5 micrograms Hg

(ml. dithizone used \times 2.5) - ml. Hg std used = micrograms Hg

(2 ml. \times 2.5) - 2 ml. = 3 micrograms Hg in digested sample

Interpretation:

1. Normal urine, blood and tissue should contain no mercury.

2. As little as 3 grams of corrosive sublimate has been found to be fatal to a child and an adult.

3. The minimum lethal dose is about 500 mg. of mercuric chloride. Organic mercury compounds vary widely in toxicity.

LEAD

Blood, Tissue and Urine

Reference:

Sandell, E. B.: Colorimetric determination of traces of metals. New York, N. Y., Interscience Publishers, Inc. 2nd Ed. (1950).

Principle:

Lead is extracted from digestion mixtures by repeated shakings with dithiozone chloroform solution. The chloroform lead dithizonate thus formed is extracted with dilute nitric acid to transfer lead to the aqueous phase. The solution is then brought to a suitable pH by adding an ammoniacal citrate cyanide solution, and the lead is again extracted with a portion of chloroform containing a known concentration of dithiozone. The concentration of lead dithizonate is found by spectrophotometric measurement of the absorption at 510 mμ and referring the results to a standard curve which has been established under the same conditions.

Reagents:

1. Metal-free water: Distilled from all pyrex glass still. (Make all reagents from this water. Wash all glassware used in procedure with concentrated nitric acid and then rinse with this water.)
2. Concentrated nitric acid: ACS-Baker's redistilled.
3. Concentrated sulfuric acid: Analytical reagent grade.
4. Chloroform: ACS-redistilled.
5. Dithiozone: 100 mg. diphenylthiocarbozone per liter chloroform.
 - a. Solution A: Make 1:1 dilution of stock.
 - b. Solution B: Make 1:10 dilution of stock.
6. Concentrated ammonium hydroxide: Analytical reagent grade. (Low lead content-0.0001%).
7. Dilute nitric acid: Dilute 10 mls. concentrated nitric acid to 1 liter.
8. Ammonium citrate: 50 grams diluted to 100 mls. with water. Make solution ammoniacal (pH-9) and shake with dithiozone until lead free. Remove excess dithiozone by shaking several times with chloroform.
9. 10 per cent potassium cyanide.
10. 20 per cent hydroxylamine hydrochloride.
11. Ammonia-cyanide mixture: 20 grams potassium cyanide and 150 mls. concentrated ammonium hydroxide diluted to 1 liter. Adjust pH to 9.5.
12. 0.100 per cent standard lead solution: 0.160 grams dried lead nitrate diluted to 100 mls. with water acidified with nitric acid.
14. Working lead standard: dilute 1 ml. of stock standard to 1 liter. (Equivalent to 1 microgram per ml.).

Procedure:

1. Collection of specimen:

a. Blood: 50 mls. are drawn with regular syringe and needle, but immediately transferred to a fifty ml. centrifuge tube that has been previously washed with concentrated nitric acid and metal free water. Use 5-10 mls. serum for each determination.

b. Urine: 24-hour specimen is voided directly into a 2 liter pyrex, glass stoppered bottle that has been previously washed with concentrated nitric acid and metal-free water. Use 100 ml. urine for each determination.

2. Digestion:

a. Samples of the specimen should be run in duplicate when possible.

b. Add 10 ml. of concentrated nitric acid to each sample and digest to about 10 ml. Add 2 ml. concentrated sulfuric acid and continue digestion until sulfur trioxide fumes are given off. (If charring occurs add more nitric acid as needed to clear). Add 3 ml. water to each sample three times, digesting to fumes between each addition.

c. A standard (10 ml. working standard) and a reagent blank (10 ml. metal-free water) are taken through digestion process. Exact quantities of all reagents are added to all digestion flasks.

3. Isolation of lead:

a. Rinse digest into a glass-stoppered separatory funnel with three 5 ml. portions of ammonium citrate.

b. Add 1 ml. hydroxylamine hydrochloride and five drops thymol blue indicator.

c. Add concentrated ammonium hydroxide until solution is basic to litmus. Add 5 ml. potassium cyanide and adjust pH to blue color of indicator with ammonium hydroxide.

d. Add 2-5 ml. dithizone solution A and shake for 15 seconds. Draw off dithizone into a separatory funnel and continue to extract until there is no mixed color formed.

e. Shake the combined extracts with 10 to 20 ml. water containing 1 drop of 50% ammonium hydroxide. Draw off dithizone and extract the ammonia solution with 1 to 2 ml. of dithizone A and add this to the main extract.

f. Shake the dithizone extract with 15 ml. dilute nitric acid for 30 seconds and transfer the aqueous phase to a separatory funnel. Extract again with 10 ml. dilute nitric acid and combine the aqueous portions. Shake several times with chloroform to remove excess dithizone.

g. Add 5 ml. ammonia-cyanide mixture and 20 ml. dithizone solution B. Shake for 1 minute. Read a portion of the dithizone layer with a spectrophotometer at a wavelength of 510 millimicrons using 10 x 75 mm. square cuvetts, and using dithizone solution B as the blank.

Calculations:

1. Per cent transmittance of the 2 unknowns, reagent blank, and the standard are compared with and read in micrograms of lead from standard curve. Report as mg. per cent lead.

Example:

- a. Per cent transmittance unknown number 1 = 95 = 2.5 micrograms
- b. Per cent transmittance unknown number 2 = 94.5 = 3 micrograms
- c. Per cent transmittance reagent blank = 99 = 1 microgram
- d. Per cent transmittance standard = 78 = 11 micrograms
- f. Knowing that the reagent blank read 1 microgram and that the standard originally contained 10 micrograms, subtract 1 microgram from the average of the 2 unknowns, = 1.75 micrograms. This is the amount of lead in the sample taken.

Standardization of Curve:

1. A series of dilutions from 5 - 10 - 15 through 50 micrograms are prepared from the stock standard. These are taken through the entire procedure and the results are plotted.

Notes:

1. All reagents used in procedure must be prepared from metal-free water.
2. All glassware used in procedure must be washed with concentrated nitric acid and rinsed thoroughly with metal-free water. Traces of nitric acid remaining in glassware will destroy the lead dithizonate color complex.

Interpretation:

1. Normals:

- a. Urine: 2.5 to 3.5 micrograms per 100 mls.
- b. Whole Blood: 0.001 to 0.006 mg. per 10 ml. sample.
 - (1) Blood cells: 0.002 to 0.11 mg. per 10 gram sample.
 - (2) Blood serum: 0.000 mg. per 10 ml. sample.

2. Acute lead poisoning:

- a. Urine: up to about 300 micrograms daily.
- b. Whole blood: in lead poisoning symptoms begin at 30 micrograms per 100 ml. with severe symptoms above 60-100 micrograms per 100 ml.

BARBITURATES

Blood, Urine, Tissue

Reference: Goldbaum, Leo R., Anal. Chem. 24, 1604-1607 (1952)

Principle:

This procedure is a simple, rapid, ultraviolet spectrophotometric procedure for the specific identification and quantitative determination of micro quantities of barbiturates. It is based on the observation that barbiturates have characteristic absorption bands in strong alkali and different characteristic absorption bands at a pH of approximately 10.5. A barbiturate is indicated only when a maximum positive difference appears at 260 mu. which decreases to a negative difference at about 250 mu. A quantitative estimation can be made from the difference at 260 mu. The variations in the optical density differences at 260 mu. due to normal absorbing substances will produce less than a 5% error. As the barbiturate concentration decreases the error will increase. As little as 1.0 microgram of barbiturate can be detected per milliliter of blood and 3.3 micrograms per gram of tissue.

Apparatus:

Beckman DU quartz photoelectric spectrophotometer, one centimeter quartz cuvettes, Waring blender, pH meter.

Reagents:

1. **Borate Buffer.** Dissolve 12.369 grams of boric acid and 14.911 grams of potassium chloride in water and dilute to 200 milliliters to prepare a 1 molar solution of these salts. Filter off any undissolved salts after standing at room temperature for 24 hours.

2. **Sodium hydroxide solutions.**

a. Prepare an approximately 0.45 normal sodium hydroxide solution. The normality is adjusted with a pH meter until a pH of 10.5 is obtained when two parts of alkali are added to one part borate buffer.

b. Prepare an approximately 0.90 normal sodium hydroxide solution from saturated sodium hydroxide. The normality is adjusted with a pH meter until a pH of 10.5 is obtained when two parts of alkali are added to two parts borate buffer.

3. **Solvents.** Wash reagent grade chloroform successively with 1 normal sodium hydroxide and twice with water. For each liter of solvent, use 100 milliliters

of wash solution. Wash only the volume required for daily use. On standing chloroform tends to decompose.

4. Standard solution. Prepare a standard solution of phenobarbital which contains 20 micrograms per milliliter.

5. Phosphate buffer. Prepare a 0.1 molar phosphate buffer with a pH of 7.4. Mix 39.50 ml. 0.1 N NaOH with 50 ml. 0.1 M KH_2PO_4 and dilute to 100 ml. in a volumetric flask.

Procedure:

A. BLOOD or PLASMA

1. Oxalated blood or plasma is adjusted to pH 4 to 5 with 0.1 N HCl and extracted with chloroform in a separatory funnel. Use 50 ml. chloroform for samples of 1 to 5 ml. Use 75 ml. chloroform for samples of 5 to 10 ml.

2. Prepare a standard control using the same volume of specimen plus one ml. of the phenobarbital standard solution.

3. Filter the chloroform extract through No. 41 Whatman filter paper. A clear aliquot of 40 to 60 ml. is obtained.

4. Extract the filtrate with 4 ml. 0.45 N NaOH using a dry separatory funnel.

5. Discard the chloroform layer and collect the alkali layer in a small tube.

6. Centrifuge the alkali extract and transfer 3 ml. of clear solution to a 1 cm. quartz cuvette.

7. Take optical density readings at 230, 240, 250, 260, 270, 280, and 305 μ against a blank of the alkali.

8. Take 2 ml. of the alkaline extract from the cuvet and mix with 1 ml. of borate buffer. The final pH should be between 10.2 and 10.6.

9. Transfer the buffered extract to a cuvette and take optical density readings at the wave lengths indicated in (7) above. The blank is prepared by mixing 2 ml. of alkali and 1 ml. of borate buffer.

10. These last optical densities are corrected for dilution with the buffer by multiplying by 1.5 and then subtracted from the optical density of the alkaline extract.

B. URINE

1. Take 1 to 5 ml. of urine with the pH adjusted to between 4 and 5 with 0.1 N HCl and extract with 50 ml. of chloroform.
2. To remove interfering substances, shake the chloroform extract with 5 ml. 1 M phosphate buffer of pH 7.4 using a clean separatory funnel.
3. Filter the chloroform layer through a Whatman #41 filter paper.
4. Take the filtrate and proceed as under BLOOD.

C. TISSUE

Tissues are prepared for extraction by homogenizing with a minimum amount of distilled water in an all-glass Waring type blender. Chloroform is the solvent of choice for all tissues except brain, where ether or ethylene dichloride can be used. A weighed sample of tissue may be homogenized and made up to a specified volume, using an aliquot for analyses; or a small sample may be homogenized and quantitatively transferred into a separatory funnel containing the solvent. For the extraction of 5 ml. of homogenate, containing 1 or 2 gram sample, 75 ml. of solvent are used. The analysis is continued as described for blood except that the aliquot of clear filtered solvent is extracted with approximately 0.9 N sodium hydroxide. The normality of the alkali is increased to eliminate the turbidity that sometimes occurs with weaker alkali. The adjustment of pH is made by adding an equal volume of boric acid-potassium chloride buffer to yield a pH between 10.5 and 10.2. The optical densities of the buffer solution are multiplied by 2. to correct for dilution with the buffer, then subtracted from those of the strong alkali.

Calculations:

$$\text{Micrograms of barbiturate per ml. or gm.} = \frac{a \times b}{c \times d}$$

- a. Micrograms of barbiturate added to known standard sample.
- b. Optical density difference of unknown extract in alkali and in pH 10.5 at 260 mu.
- c. Amount of sample.
- d. Optical density difference of standard extract in alkali and pH 10.5 at 260 mu. minus (b) above.

EXAMPLE:**Optical Density Readings**

Wave length	Standard Control				Unknown			
	Alkaline	pH 10.5 buffer	x 1.5	Difference	Alkaline	10.5	x 1.5	Difference
230	.670	.392	.588	.082	.800	.498	.748	-.052
240	.470	.428	.552	-.082	.600	.490	.735	-.135
250	.470	.307	.465	.005	.540	.361	.542	-.002
260	.465	.184	.276	.189	.495	.259	.388	.107
270	.380	.170	.255	.125	.412	.247	.370	.042
280	.305	.171	.257	.048	.350	.243	.365	-.015
290	.250	.153	.230	.020	.293	.210	.315	-.022
305	.130	.082	.123	.007	.160	.110	.165	-.005

a 20 micrograms

$$b \quad .107 \quad \frac{20 \text{ ug} \times .107}{5 \text{ ml.} \times .082} = 5.22 \text{ ug/ml.}$$

c 5 ml. blood

$$d \quad .189 - .107 = .082$$

NOTES:

1. The optical density at 305 mu is an indication of the amount of absorbing substances other than barbiturates. The differences at this wave length should be insignificant.
2. The sample used for analysis should be such that the alkaline extract should contain about 25 micrograms of barbiturate per ml.
3. Salicylates do not interfere.
4. Dicoumarol, dilantin, and sulfadiazine appear in the alkaline extract but do not interfere because they have the same absorption in alkali and in pH 10.5 solution.
5. The sodium hydroxide solutions can be stored in polyethylene bottles without decomposition.

Interpretation:

After a therapeutic dose of a barbiturate the blood level may be 1 to 10 micrograms per ml. The corresponding tissue level is higher. The toxic level ranges from 15 to 100 micrograms per ml. The effective dose of barbiturates varies greatly with the particular compound used, as does also the effective concentration in tissues. Since the toxic level of one barbiturate may be a safe level for another, it is important to be able to identify the particular barbiturate in question. A paper chromatographic method of identification is given in this manual p. 292.

PAPER CHROMATOGRAPHY FOR THE IDENTIFICATION OF THE COMMON BARBITURATES IN BLOOD, URINE, AND TISSUE

Reference:

Algeri, E. J., and Walker, J. T.: Am. J. Clin. Path., 22, 37-40 (1952).

Principle:

A paper chromatographic method is used for the specific identification of the common barbiturates. This technic is used in conjunction with the spectrophotometric procedure previously outlined. For a given solvent and temperature, a substance has a characteristic R_f value; its determination serves as a means of identification.

Apparatus:

1. Museum jars approximately 18 inches high and 8 inches wide equipped with rubber gasket, lid, and screw clamp. Stopcock grease may be used as an air seal.
2. Ultra-violet lamp.
3. Whatman No. 1 filter paper 17 x 14 inches.

Reagents:

1. Solvent - n-Butyl alcohol is saturated with 5 Normal ammonium hydroxide by shaking in a separatory funnel and allowing the two phases to separate. The aqueous portion is then drawn off into a small beaker and placed in the bottom of the tank, separate from the surrounding saturated mobile organic phase. This solution should be freshly prepared.

2. Color developer

- a. Silver acetate - A 0.1 per cent aqueous silver acetate solution is prepared.
- b. s-Diphenyl carbazone - A 0.1 per cent s-diphenyl carbazone solution in absolute alcohol.

3. Standards - Standard barbiturate solutions are made up in acidified water (1:500 H_2SO_4) to contain 1.0 milligrams per milliliter.

Procedure:

1. The combined alkaline extracts containing a barbiturate (from the quantitative procedure, p. 288) is acidified with dilute hydrochloric acid and extracted at least twice with ether.
2. The ether extract is reduced in volume by evaporation.
3. Approximately 0.01 ml. of the extract is applied to the filter paper cylinder at a base line 3 cm. from the bottom.

4. Apply 0.01 ml. (10 microgramm) of the known barbiturate standards at 3 cm. intervals to the paper.

5. Roll the paper cylindrically and staple so that the edges do not touch.

6. Hang the paper cylinder in the tank containing the solvent for one hour.

7. Lower the paper cylinder into the solvent.

8. Place the system in a dark cabinet at constant temperature for 4 hours. The solvent front should move at least 120 mm.

9. Dry the chromatogram rapidly and uniformly in air to minimize the spreading of the spots. The staples are removed.

10. Locate the spots by placing a pencil dot in the center of the dense area, while observing under ultra-violet light.

11. The sheet is dipped in 1 per cent silver acetate solution for one-half minute, washed four minutes in running water, and immersed directly in 0.1 per cent α -diphenyl carbazone in 95 per cent ethyl alcohol for one minute. The spots appear deep brown in color against a light pink background.

12. Determine the R_f values for each spot.

Calculations:

$$R_f = \frac{\text{Distance moved by solute}}{\text{Distance moved by solvent}}$$

Example:

Barbiturate	Distance of solute	\div	Distance of solvent	=	R_f
Seconal	112 mm.		132 mm.		.886
Barbital	78		131		.596
Unknown	83		131		<u>.634</u>
Phenobarbital	83		130		<u>.639</u>
Amytal	109		131		.832
Neimbutal	100		131.5		.756

The R_f value of the unknown after four hours running time coincides with that of phenobarbital.

Notes:

1. Pentothal is revealed under ultra-violet light without being developed.

SIMPLE BLOOD TESTS AVAILABLE TO THE GENERAL PRACTITIONER

Reference:

Mandel, E. E., M.D., Lehmann, E. B., M.D., Paris, D. A., M.D., U.S. Public Health Service. Communicable Disease Center, Federal Security Agency. March 1950.

Blood Tests:

Sedimentation Rate
Icterus Index
"H" Flocculation
Creatinine
Glucose

Reagents:

Potassium ammonium oxalate solution:

Ammonium oxalate 1.2 g., potassium oxalate 0.8 g., distilled water, dilute up to 100.00 cc. Use 2 drops (dried) for each ml. of blood.

Saturated solution of picric acid prepared at 24° C.

10% sodium hydroxide.

Distilled water.

Hayem's solution - Mercury bichloride 0.25 gm., sodium sulfate 2.50 gm., sodium chloride 1.00 gm., distilled water, dilute up to 100.0 cc.

Apparatus:

12 pyrex test tubes, without lip, 100 x 13 mm., graduated at 1, 2, 4, and 5 cc., and several dozen identical test tubes, nongraduated.

Test tubes of identical size containing standard solutions of potassium dichromate, all stoppered and sealed with paraffin.

Small funnels and filter paper (Whatman No. 1).

Medicine droppers.

Beaker or small pot, stand and bunsen burner or electric hot plate for water bath.

3-slot comparator with ground-glass background, approximately 3 x 2 1/2 x 1 inch in dimension.

Desk lamp with daylight bulb

Set of Westergren sedimentation tubes.

Principle:

A knowledge of the physiological basis for the importance of these tests will be assumed, and only the procedures will be described. Routine analysis of the blood and

urine is an invaluable adjunct to the clinical examination. Yet the equipment, time and technical skill required for many standard blood tests make their performance in the average practitioner's office impractical. In addition, a clinical laboratory may be too distant or its fees too high for the patient to afford. Therefore, it has seemed highly desirable to find methods that are "so simple and mechanical in their operation that the possibility of large error is excluded and so direct that the results are presented as pictures whose significance can be read at a glance." If such tests could be performed by the doctor or his nurse with little loss of time, the results would constitute a valuable supplement to the physical examination, helpful both in the ready detection of disease and in the follow-up of patients.

A. Collection of Blood Samples: The five simple blood tests to be described, used either singly or as a "battery," or in combination with cell count and other procedures, will provide quick and valuable diagnostic information with a minimum of equipment, expense and technical experience. They can be all carried out on a single sample of venous blood. Only about 7 cc. is required, this being divided into two parts. Five cc. is allowed to coagulate in one tube to provide serum for Hayem's test and Icterus Index, and 2 cc. is mixed in another tube with the dried residue of four drops of oxalate solution for the sedimentation rate, creatinine and glucose determination, and also for blood count if desired.

B. Sedimentation Rate: Using the Westergren tube as a pipette, oxalated blood is sucked up to the zero mark. At the end of one hour the height of the boundary between the packed cells below and the supernatant plasma above is read from the calibrations on the tube. A drop of the level of the cell column of more than 10 mm. in men or more than 15 mm. in women in one hour is suggestive of disease.

Erroneous results may be obtained if there is prolonged venous stasis on withdrawing blood, traces of water, alcohol, ether and other substances in syringe, needle or tube, or delay of over six hours in conducting the determination. Results will also be affected by exposing the specimen to temperatures considerably above or below average room temperature, and by racking the tube in a position that is not exactly perpendicular.

C. Creatinine: The urine and blood levels of creatinine are essentially independent of the amount of protein consumed and the time of food intake. This is in contrast to urea, the blood concentration and urinary elimination of which are greatly influenced by these physiologic factors.

To 4 cc. of a saturated solution of picric acid add 1 cc. of oxalated blood, using syringe or medicine dropper. Shake and let stand 5 minutes. Filter into an identical test tube until the clear yellow filtrate reaches the 1 cc. mark. Add 1 drop NaOH. Shake. In 15 minutes compare with standard solutions. An increase of 3 mg. per cent or more can be detected readily without the aid of a comparator.

D. Glucose: Same solution that has been used for the creatinine determination is immersed in boiling water for about 3 minutes. Distilled water is added to the 5 cc. mark. Shake and compare with the standard solutions. If color is more intense than 2400 mg. per cent dichromate standard, transfer 1 cc. to another tube and dilute with

1 cc. distilled water. After dilution the value obtained by color comparison must be doubled to obtain actual glucose level. Further dilution to the 4 cc. mark is necessary if blood sugar is above 400 mg. per cent.

A coincidental rise in creatinine increases the intensity of the picrate glucose color reaction sufficiently to warrant its consideration in blood specimens containing up to 200 mg. per cent of glucose. For every milligram per cent of creatinine found in excess of the average normal of 1 mg. per cent, 2 mg. per cent of glucose should be deducted from the final glucose value. This may well be ignored, however, for the sake of convenience and in such undertakings as screening for diabetes since the coincidence of azotemia and diabetes is infrequent and the error would be on the plus side: it would tend to add an occasional uremic individual to the hyperglycemic group but would not cause any diabetics to be missed.

The visual tests for creatinine and glucose have been found useful as ready checks on doubtful results of the precision methods. They are suitable for front-line practice in the absence of routine laboratory facilities and they afford information that is adequate for many clinical purposes.

E. Icterus Index: The icterus index may be determined by direct comparison of the color of either plasma (oxalated blood) or serum (coagulated blood) with the tubes containing standard solutions of potassium dichromate. Intensity of the yellow color of the sample in excess of a 70 mg. per cent dichromate solution is an abnormal finding. The test is of value in detecting abnormal amounts of blood bilirubin even in the absence of clinically apparent jaundice, without differentiating between the various types of jaundice.

F. Hayem's "H" Test: The finding of serum protein alterations is indicative of organic disease. The various flocculation tests in general use constitute, in essence, practical qualitative substitutes for the exact measurement of the various serum protein components by chemical, electrophoretic and other means. While used largely as "liver function tests", they may be employed advantageously in other diseases, especially those of infectious or neoplastic origin. Positive reactions usually are taken to reflect hypoalbuminemia and/or an increase in one or more globulins.

The flocculation test with Hayem's solution is one of the simplest and quickest in this category. As a nonspecific screening test it was found to be more sensitive than the Takata-Ara, cephalin-cholesterol flocculation, and formol-gel reactions, and about equal to the thymol turbidity test; it was not positive as often as the sedimentation rate and the zinc sulfate turbidity method. In testing for primary hepatic disease, the "H" reaction was surpassed in sensitivity only by the sedimentation rate, thymol and zinc sulfate turbidity tests. It was positive in all examined cases of severe or prolonged hepatitis of hepatic cirrhosis, and of active rheumatoid or infectious arthritis; furthermore it was positive in 51 per cent of acute infections, 47 per cent of chronic lung disorders and 59 per cent of cancer.

A positive "H" flocculation implies systemic disease (metabolic, infectious or neoplastic) usually of a more serious and/or advanced nature than that indicated by an increased sedimentation rate. It may be the first abnormal finding in such conditions as cirrhosis, syphilis, pyonephrosis, tuberculosis or metastatic neoplasm. The test has been found helpful in such problems of differential diagnosis as obstructive vs. hepatic

jaundice, tumor of the right kidney vs. hepatomegaly, epistaxis due to local lesion vs. chronic hepatic disease, cholecystopathy vs. neoplastic liver involvement, "rheumatism" vs. rheumatoid arthritis, congestive vs. infectious pleural effusion, nutritional vs. leukemic anemia. In each of these cases a positive reaction favors a hepatic, infectious or neoplastic cause of the condition.

The Hayem test may serve as a prognostic adjunct to the sedimentation rate and as a practical substitute for more elaborate tests in the screening for, and management of, diseases affecting the reticulo-endothelial system.

Procedure: Equal volumes of serum (from coagulated tube) and Hayem's solution - usually 1 cc. of each - are mixed. Development of a precipitate within 1 to 24 hours is considered a positive reaction (graded as 1, 2 or 3+). An immediate prediction of the eventual outcome can usually be made by overlaying the serum with 1 drop of Hayem's solution. A faint transient clouding may occur in normal serum, but a well discernible "reversible flocculation" is abnormal.

BLOOD VALUES CORRESPONDING TO EACH OF THE STANDARD SOLUTIONS

Potassium																			
Dichromate %	.05	.07	.10	.15	.25	.30	.50	.70	.90	1.0	1.2	1.5	1.9	2.4	3.0				
Glucose mg. %				50	78	90	112	130	145	150	160	175	185	200	215				
Creatinine mg. %				0.8	1.6	2.0	3.2	4.2	5.0	5.4	6.0	7.1	8.0	9.8	14				
Icterus Index units	5	7	10	15	25	30	50	70	90	100	120	150	190	240	300				

ROUTINE CHEMICAL ANALYSIS OF URINARY CALCULI

Reference:

Winer, J. H., Mattice, M. R.: Routine Analysis of Urinary Calculi. A simple rapid method using spot tests. *J. Lab. & Clin. Med.* **28**, 898-904 (1942).

Principle:

After preparing a physical description of the calculus as to weight, size, shape, color, surface appearance, and consistency, the calculus is finely pulverized in a mortar for chemical analysis. This analysis is performed using a series of black and white spot plates, and the characteristic groups tested for by means of simple spot tests.

Apparatus:

- 1 12-hole white spot plate
- 2 3-hole black spot plate

Reagents:

1. Sodium Carbonate: Dissolve 20 grams of the anhydrous salt in 80 ml. water.
2. Uric Acid Reagent: Dissolve 10 grams pure sodium tungstate (Na_2WO_4) in about 60 ml. water; add 5 grams pure arsenic pentoxide, 2.5 ml. of 85% phosphoric acid, and 2 ml. concentrated HCl. Boil mixture 20 minutes, cool, and dilute to 100 cc. with water. Filter if solution is cloudy.
3. Ammonium Molybdate: Dissolve 3.5 grams of the salt $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in 74 ml. water, add 25 ml. concentrated nitric acid, and shake until dissolved.
4. Ammonium Hydroxide: Concentrated, Reagent grade.
5. Sodium Cyanide: Dissolve 5 grams in 100 ml. water, and add 0.2 ml. ammonium hydroxide as a preservative. Store solution in refrigerator.
6. Sodium Nitroprusside: Dissolve 5 grams in 100 ml. of water. Discard when color fades.
7. Nitric Acid: Concentrated, Reagent grade.
8. Ammonium Sulfocyanate: Dissolve 3 grams of the salt (NH_4SCN) in 100 ml. water.
9. Hydrochloric Acid: Add 10 ml. of concentrated HCl to 90 ml. of water.
10. Sodium Oxalate: Saturate 100 ml. of water with the salt by adding 5 grams a and allowing to settle.
11. Sodium Hydroxide: Dissolve 20 grams of the Reagent grade pellets in 80 ml. of water.
12. Titan Yellow Dye: (also called "Clayton Yellow"). Dissolve 0.1 grams of dye in 100 ml. water. Make the solution alkaline with 3 drops of 20% sodium hydroxide (see No. 11). Store in an amber bottle. Prepare fresh every 30 days.

13. Nessler's Reagent: Dissolve 22.5 grams of iodine in 20 ml. of water containing 30 grams of potassium iodide. Add 30 grams of pure metallic mercury. Shake the mixture well, cooling frequently under tap water until yellow color due to iodine disappears. Decant the supernatant aqueous solution. Test a portion of it by adding a few drops of the reagent to 1 ml. of 1% soluble starch solution. Test should be positive (a purple to black color). Dilute with water to about 200 ml., mix well, and add to 975 ml. of 10% sodium hydroxide. Mix well and allow to clear by standing.
14. Manganese Dioxide: powdered, Reagent grade.
15. Barium Chloride: Dissolve 5 grams of the salt in 100 ml. of water.
16. Sodium Nitrite: A fresh solution, 0.1 grams of the salt per 100 ml. of water, should be prepared weekly.
17. Reagent "S": Dilute 1 ml. of dimethyl- α -naphthylamine (Eastman) in 250 ml. of 95% alcohol.
18. Chloroform: pure, Reagent grade.
19. Acetic Anhydride: concentrated, Reagent grade.
20. Sulfuric Acid: concentrated, Reagent grade.

Procedure:

1. Weigh the stone on the analytical balance to the nearest one-tenth milligram. If stone has been freshly taken or has been in preservative, allow to dry at room temperature for at least 24 hours before beginning this procedure.
2. Next, examine the stone in a good light. With the aid of a hand lens note the color, shape, and surface appearance, (See notes 1-2).
3. Using a metric ruler, measure the stone's length, width, and thickness to the nearest millimeter.
4. In a small clean mortar pulverize about 50 mg. of the stone, noting its consistency. (See note 3).
5. Refer to Table 1 and first complete this routine spot test battery. Perform all spot tests on a white spot plate unless otherwise indicated in the table. (Starred (*) tests are to be performed on black spot plate). Note all positive reactions.
6. If no positives have been found in Table 1, or if one of the rarer calculi is suspected, next refer to the "Table of Special Tests," Table 2 for a more complete analysis.

TABLE NO. 1 - ROUTINE TESTS ON URINARY CALCULI

<u>Chemical Group</u>	<u>Reagents Added</u>	<u>Results</u>
1. URATES	Pulverized stone 1 drop Na_2CO_3 2 drops uric acid Reag.	Prompt deep blue color (pale blue is negative)
2. PHOSPHATE	Pulverized stone 5 drops ammonium molybdate	Bright yellow precipi- tate is positive (allow 5 minutes)
3. IRON	Pulverized stone 3 drops nitric acid 3 drops ammonium thiocyanate	Red color is positive for iron
4. CYSTINE	Pulverized stone 1 drop NH_4OH 1 drop sodium cyanide Wait 5 minutes 2 drops sodium nitro- prusside	Beet-red color is positive for cystine. (May fade to red-orange)
Dissolve 25 mg. of the remaining pulverized stone in 2 ml. of 10% hydrochloric acid in a small test tube. Stir for 2 minutes.		
5. CARBONATE	Observe the HCl solution of calculus for bubbles.	Effervescence is posi- tive for carbonate.
6. CALCIUM*	3 drops acid extract 4 drops sodium oxalate 3 drops NH_4OH	White precipitate is positive for calcium.
7. MAGNESIUM	3 drops acid extract 3 drops sodium hydroxide 1 drop Titan Yellow Dye	Blood red precipitate is positive (any other color is negative)
8. AMMONIA	3 drops acid extract 3 drops sodium hydroxide 4 drops Nessler's Reagent	Yellow-orange precipi- tate is positive for ammonium
9. SULFATE*	3 drops acid extract 4 drops barium chloride	White precipitate is posi- tive for sulfate
10. OXALATE	To the remaining acid ex- tract in the test tube add a pinch of manganese di- oxide.	Tiny bubbles of gas "ex- plosively released" is positive for oxalate.

TABLE NO. 2 - SPECIAL TESTS FOR RARER URINARY CALCULI

Chemical Group	Reagents Added	Results
1. XANTHINE	Pulverized stone Dissolve in nitric acid Dry in evaporating dish on water bath Add 3 drops ammonium hydroxide	Yellow residue which turns orange when the ammonia is added is a positive test
2. INDIGO	Pulverized stone 5 drops chloroform	Blue color is positive for indigo
3. CHOLESTEROL	Pulverized stone 5 drops chloroform 10 drops acetic anhydride 1 drops sulfuric acid	Play of color ending in green is positive for cholesterol
4. SULFONAMIDE	Pulverized stone 2 drops HCl 2 drops sodium nitrite 3 drops ammonium sulfamate 3 drops Reagent "S"	Red color is positive for sulfonamides

7. After completing the spot test analysis, report all of the physical and chemical findings in the following manner.

NAME John Doe WARD 126 DATE 12/10/57

Source of Calculi: Renal

Weight 67.5 mg.

Size 5 x 4 x 3 mm.

Shape round

Color red-brown

Surface Appearance roughened

Consistency hard, brittle

Chemical Analysis Ca, Mg, CO₃, C₂O₄

Category of Stone OXALATE-CARBONATE TYPE

NOTES:

1. The shape, weight, and size of a particular calculus is quite independent of the type of stone. In reporting shape the calculus is described as being either oval, round, flat, cubic, pyramidal, or irregular.
2. The color, surface appearance, and consistency of the calculus are closely related to the type of stone. Listed in Table 3 are the most common characteristics of various types of calculus. A study of the physical characteristics is often helpful in confirming a doubtful positive chemical test.

TABLE NO. 3 - PHYSICAL CHARACTERISTICS OF CALCULI

Type of Calculus	Color	Surface Appearance	Consistency
Urate	yellow to dark red-brown	slightly rough	quite hard
Phosphate	white, gray, to yellow-gray	smooth, sandy	chalky, brittle
Cystine	pale yellow	waxlike	soft
Carbonate	dull white to red-brown	chalky	usually quite hard
Oxalate	white, grey, or red-brown to black	tuberculated or quite rough	very hard
Xanthine	brown	smooth, wax-like	soft

3. In about 40% of the stones submitted for analysis, the quantity is not sufficient to provide 50 mg. of material for analysis. Therefore it is best to test for the presence in these cases of calcium, phosphate, and oxalate first, before proceeding with the other tests. Stones weighing less than 5 mg. are almost impossible to analyze correctly by this method.

**TABLE NO. 4 - FREQUENCY OF OCCURENCE OF THE VARIOUS
CHEMICAL CONSTITUENTS IN CALCULI***

<u>Chemical Group</u>	<u>% Occurrence</u>
Calcium	97%
Phosphate	88%
Oxalate	65%
Urate	15%
Carbonate	12%
Magnesium	25%
Ammonium	20%
Cystine	2%
Iron	Rare
Sulfate	Rare
Xanthine	0.5%
Cholesterol	Rare
Indigo	Rare
Sulfonamides	Rare

***Based upon a study of calculi analyzed by this laboratory through the
year of 1956.**

CHOICE OF LABORATORY TESTS

Reference: Perrin H. Long, M.D., Modern Medicine, November 1, 1954.

Excessive reliance on laboratory tests is expensive for the patient and, by overloading the technician with work, impairs accuracy in the laboratory. Physical examination and determination of previous disease of the patient often obviates the need for many laboratory procedures.

Hemoglobin, leukocyte count, and differential count from a stained smear should be determined at the initial examination of a patient. The sedimentation rate is measured, and the feces are tested for occult blood with guaiac. Sickle-cell preparations are made for new Negro patients. Urinalysis should include specific gravity, albumin, sugar, and acetone measurements and microscopic examination of the sediment.

Serologic reactions for syphilis are often done at the first examination. Spinal fluid of patients with syphilis is studied at least once.

Blood urea nitrogen or nonprotein nitrogen is measured in a patient with albuminuria, progressive hypertension, changes in the retinal arterioles, or an enlarged prostate. The fasting blood sugar is determined for patients with glycosuria or suspected hypoglycemia and as an aid in the management of diabetes.

Knowledge of serum electrolyte concentrations is valuable with dehydration, vomiting, and diarrhea. The carbon-dioxide combining power of the blood and serum levels of chlorides are helpful in treatment of diabetic acidosis, pulmonary insufficiency, and severe renal disease. Sodium concentration is measured when Addison's disease is suspected and during therapy by salt restriction and diuretics. Potassium levels should be determined when parenteral fluids are injected or when gastric suction is continuous, and for certain types of renal insufficiency.

In the differential diagnosis of jaundice, the serum bilirubin is more valuable than the icterus index, and the alkaline phosphatase level provides additional help. The thymol turbidity is more useful than the cephalin flocculation in assessing hepatocellular damage. Bromsulphalein retention is accurate when the patient is not jaundiced.

Total proteins are measured in the study of malnutrition, anemia, ascites, and edema. Determination of the albumin and globulin should be deferred until the total proteins are known, unless multiple myeloma is suspected.

Use of laboratory procedures should be limited to those specifically indicated.

CLINICAL INTERPRETATION

<u>Determination</u>	<u>Normal Range</u>	<u>Discussion of variations in Disease States</u>
Amylase (serum)	50-200 units/100 ml.	Increased values are seen in diseases involving organs secreting amylase. For example; acute and chronic pancreatitis; secondary pancreatitis; and mumps.
Basal Metabolic Rate	-10 to +15% of average normal	<p>BMR is elevated in:</p> <ol style="list-style-type: none"> 1. High calorie diet; 2. Fever; 3. Hyperthyroidism; 4. Other endocrine disorders. <p>The BMR is decreased in the opposite conditions. In addition, apprehension or nervousness can elevate the results 20-30% above the true value in the absence of disease.</p>
Bilirubin (serum)	1'B below 0.25 mg. % TB below 1.50 mg. %	<p>Hemolytic jaundice - Total bilirubin is increased but the fraction reacting promptly (1' B) is not.</p> <p>Obstructive and Hepato-cellular Jaundice - Both fractions are increased.</p>
Bromide (serum)	Less than 3 mg. % (non-specific color)	Increased in Bromide Intoxication, clinical signs of which are usually evident at serum levels of 100-200 mg. %.
Bromsulfalein Test	<p>Below 10% at 30 min. Below 7% at 45 min. 0-1% at 60 min. Clearance 5.33/0.75 ml. /min. /kg.</p>	<p>This test is most useful in liver disease without jaundice. In advanced cirrhosis 50% may be retained after 45 minutes. This is one of the most useful liver function tests.</p>
Calcium (serum)	9 to 11.5 mg. %	<p>Serum calcium may be altered in the following ways: Deficient absorption (Vitamin D deficiency) usually results in a low normal serum calcium. Diseases of the parathyroid gland may cause increases up to 20 mg. % in parathyroid excess; decreases (below 6 mg. %) in deficit. Serum calcium also varies inversely with serum phosphate (for example in terminal uremia). With phosphate</p>

<u>Determination</u>	<u>Normal Range</u>	<u>Discussion of Variations in Disease States</u>
Calcium (cont.)		retention, calcium will fall). Changes in plasma proteins will cause change in total serum calcium since part of the calcium is "bound" to the protein.
Calcium (urine) "Sulkowitch test"	Moderate	Urine results reflect (ionized) serum calcium. No precipitate means a total serum calcium less than 8.5 mg. %. A fine cloud means serum calcium is normal. A heavy cloud means a total serum calcium of more than 12.5 mg. %.
Cephalin Flocculation - see "Thymol Turbidity"		
Chloride (serum)	As NaCl 555-620 mg. % As Cl⁻ 340-375 mg. % 95-106 mEq. /l.	For variations in disease see Acid-Base Equilibrium (Appendix). Serum Cl ⁻ is lowered during excessive loss of Cl ⁻ as in diarrhea and vomiting and in some infectious diseases such as meningitis and pneumonia. Increases are rare and poorly understood.
Cholesterol Cholesterol Esters	125-250 mg. % 60-75% esters	Increases in nephrosis (600-700 mg. %) diabetes mellitus, obstructive jaundice, hypothyroidism (500-700 mg. %). Decreases are seen in hyperthyroidism (80-100 mg. %) and some other infections, wasting diseases and anemias. In liver diseases, the % due to cholesterol esters decreases (down to 10%).
Congo Red Test	More than 60% retention	This is a test for "amyloid disease". Deposits of "amyloid" in tissue absorb congo red. In amyloid disease less than 40% of the dye will remain in the plasma. In extreme cases as little as 10% may remain.
Creatine (urine)	0-200 mg. /24 hr.	Urine creatine increases in nuclear disorders of muscle, starvation, fevers, etc., especially in muscular dystrophy.
Creatinine (serum) (urine)	0.7-1.4 mg. % 1-2 g. /24 hr.	Serum creatinine increases in renal disease, but later than does urea, and more than 5 mg. % in a chronic nephritis

<u>Determination</u>	<u>Normal Range</u>	<u>Discussion of Variations in Disease States</u>
Creatinine (cont.) (serum) (urine)		indicates a poor prognosis. Creatinine clearances are becoming increasingly used for renal function studies.
Fibrinogen	0.113-0.380 mg. %	Fibrinogenopenia (decreased fibrinogen) may be congenital or acquired, by <u>in vitro</u> clotting or by severe hepatic dysfunction. Increases are seen in most infectious diseases except typhoid fever.
Glucose (blood)	90-120 mg. % 60-80 mg. %	Total reducing substances. True glucose. Increases are seen in diabetes mellitus up to 500-1000 mg. %. Decreases in insulin shock to 20 mg. % or lower.
Glucose tolerance		See the Manual for Interpretation p. 184.
Icteric Index	4-6 units	7-15 units may be measured in latent (sub-clinical) jaundice. Over 15 results in obvious jaundice. Increases are seen in many types of liver and gall bladder and bile duct disease as well as in hemolytic disorders. Carotenes (pro-vitamins A) may contribute to the color of the serum as well as the more usual bilirubin (see BILIRUBIN).
Kepler-Power Water Test	See under this heading in Manual, p. 85.	
Lead (urine)	30-80 ug/24 hr.	In acute lead poisoning up to 300 ug/day. See PORPHYRINS (urine).
Sulkowitch Test	See CALCIUM (urine)	
Nitrogen Non-Protein (Blood NPN)	25-35 mg. %	Since NPN is composed largely of urea, creatinine, and uric acid (along with amino acids) the same sort of interpretations arise. See under these listings.
Phosphate (serum)	2.5-4.5 mg. % (adult) 4.0-6.0 mg. %	<u>Increases:</u> Chronic nephritis up to 20 mg. % Hypoparathyroidism up to 7 mg. % <u>Decreases:</u> Hyperparathyroidism to 2 mg. %

<u>Determination</u>	<u>Normal Range</u>	<u>Discussion of Variations in Disease States</u>
Phosphate (serum) (cont.)		Insulin injection or glucose tolerance test to below 3 mg.%. During treatment of diabetic coma the value may fall to below 1 mg.%. In general, serum phosphate and serum calcium levels rise and fall reciprocally.
Phosphatase (serum) (Bodansky units)	1.5-4.0 units/100 ml. (adults) Up to 12 units/100 ml. (child)	Increases are seen during actual or attempted bone regeneration. Rickets: up to more than 100 units Paget's Disease of Bone: up to 500 units Hyperparathyroidism: 20-700 units In liver disease the phosphatase of serum may be raised because of failure of the normal excretion of this enzyme in the bile.
Porphyryns (urine)		
Porphobilinogen	Negative	Found present in acute idiopathic porphyria along with uro-porphyrins (absent in pure cutaneous types).
Uro-porphyrins	Less than 10 ug/day	Ordinary tests are interpreted as normally negative. In the rare congenital porphyria, large amounts (50-100 mg./day) are excreted, mainly Type I.
Coproporphyrins	50-250 ug/day	Coproporphyrin I and III are normally present in urine. They are increased in liver disease (up to 1 mg./day), heavy metal poisoning (5-10 mg./day).
Porphyrin precursors are present to a variable extent. Porphobilinogen is only one of several precursors, whose presence may be demonstrated by appropriate treatment (such as heat, light, or I ₂) of the urine or of urine extracts.		
Sodium (serum)	134-144 mEq./l	See "Acid-Base" Equilibrium (Appendix).
Potassium (serum)	3.5-5.3 mEq./l	In general excessive loss of body fluids decreases serum Na, which is also low in Addison's disease; an increase in serum K is seen in Addison's disease; a decrease in treated diabetic coma, diarrhea and vomiting.
Protein (serum)	Total 6.5-7.5 g.% Albumin 4.0-5.5 g.% Globulin 1.7-3.0 g.% A/G ratio 1.4-3.0	Albumin: an increase in albumin occurs only in dehydration and the rise is paralleled by a rise in globulin; thus the A/G ratio is constant. A decrease in albumin

<u>Determination</u>	<u>Normal Range</u>	<u>Discussion of Variations in Disease States</u>
Protein (cont.)		is seen in (a) loss of albumin in urine; (b) decreased formation in the liver; (c) increased metabolism of protein; (d) protein starvation; (e) diversion of protein synthesis toward globulin. Globulin is increased in liver disease, multiple myeloma, and in chronic infections. The albumin is often reciprocally reduced, resulting in a A/G ratio below 1 (a "reversed" ratio). For fibrinogen, see above. For gamma globulin see zinc turbidity.
Prothrombin Time	11-15 seconds	For discussion of the clotting factors see p. 324. A prolonged prothrombin time (lowered percentage) is seen in liver disease, vitamin K deficiency, dicoumarol treatment or poisoning.
Salicylic Acid	0 - 1 mg. % (non-specific color)	In the treatment of rheumatic disease, levels of 35 mg. % may be desired. This borders on the toxic level, so close laboratory control may be required.
Sulfonamides	None	The "therapeutic level" varies but usually is between 10 and 15 mg. % but in intracerebral infections some workers have used levels of 30-40 mg. %.
Thiocyanate	None	Thiocyanate may be used for treatment of hypertension or for estimation of "total" extracellular fluid volume. Toxic levels are reached at about 12 mg. % in serum.
Thymol Turbidity	0 - 4 units	These three tests roughly parallel each other and depend on (a) increased γ -globulin; (b) decreased serum albumin; (c) qualitative changes in serum globulin, serum albumin, and phospholipids. Diseases in which these tests are usually positive are: infectious hepatitis, cirrhosis, infectious diseases with antibody production (gamma-globulin).
Zinc Turbidity	2 - 8 units	
Cephalin		
Flocculation	0 - 1 units	

<u>Determination</u>	<u>Normal Range</u>	<u>Discussion of Variations in Disease States</u>
Urea Nitrogen	8-18 mg. %	Blood urea nitrogen (BUN) is increased in: (a) pre-renal states, e.g., dehydration with reduced plasma volume and thus decreased glomerular filtration rate; (b) renal states, e.g., acute glomerulonephritis, mercurial poisoning, etc.; (c) post-renal states, e.g., obstructive lesion of ureter. Decreases in BUN may be seen in very severe hepatic failure due to failure of the liver to deaminate amino acids. Urea clearance is a more sensitive indicator of renal disease than is the BUN.
Urea Clearance	75-125 % of average normal	
	54 ml./min. = C_g	
	75 ml./min. = C_m	
Uric Acid (serum)	2 - 6 mg. %	Increases in serum uric acid are seen in: (a) renal disease 4-20 mg. %; (b) gout 5 to 10 mg. %; (c) leukemia up to 10 mg. %; (d) toxemia of pregnancy up to 20 mg. %. Uric acid clearance averages about 10% of the urea clearance, and is being used to investigate pre-eclamptic states.
Uric acid clearance	6 - 12 ml./min.	
Zinc Turbidity	See "Thymol Turbidity," above.	

URINE EXAMINATION

Chemical Tests:

- | | | |
|-------------------------|--|--|
| (a) H^+ concentration | 24 hr. - pH 6.0
Random 4.5-5.2 | Usually (but not always) the urine will tend to be alkaline in alkalosis and acid in acidosis. See "Acid-Base Equilibrium" |
| (b) Specific gravity | 24 hr. 1.016 - 1.025
Random 1.002 - 1.040 | Specific gravity varies with the balance between intake of fluid and loss by the other routes of water disposal (perspiration, vomiting, diarrhea, etc.). In diabetes insipidus, very large volumes of very dilute urine are excreted. In diabetes mellitus, glucose excretion may greatly increase the specific gravity, due to glycosuria. |

<u>Determination</u>	<u>Normal Range</u>	<u>Discussion of Variations in Disease States</u>
Chemical Tests (cont.):		
(c) Protein	Qualitatively negative	Several types of proteins may be found in urine: (a) albumin and globulin; (b) fibrinogen; (c) gross blood; (d) abnormal proteins - such as "Bence-Jones." Pathologically the protein may be due to (a) pre-renal causes such as dehydration, intra-abdominal pressure, fevers, etc.; (b) renal - all types of renal disease may cause proteinuria such as glomerulonephritis, nephrosis, etc.; (c) post-renal - addition of protein to urine after it leaves the tubules may occur from the renal pelvis on; such as ureter, bladder, urethra, etc.; (d) abnormal proteins - e.g., "Bence-Jones" protein may be excreted through the glomerulus.
(d) Reducing Substances		
"Glucose"	Less than 0.1%	Glucose is found in urine of all persons when their blood sugar is elevated above the "renal threshold" such as in (a) diabetes mellitus; (b) hyperthyroidism, pituitarism, or-adrenalism; (c) severe liver or pancreatic disease; (d) infection; (e) anesthesia or asphyxia; (f) renal glycosuria-low renal threshold as in pregnancy. May be found in urine during lactation. It may be very important to determine the exact identity of the sugar.
(e) Acetone Bodies	Negative	
Acetone		These substances are present in acidosis due to excessive fat metabolism as in untreated diabetes mellitus, and starvation.
Aceto-acetic acid		
B-hydroxy-butyric acid		
(f) Bile Pigments	Negative	Bilirubin is found in the urine in cases of obstructive jaundice and most cases of hepatocellular jaundice. It is not present in most cases of hemolytic jaundice. Urine levels may be elevated as early as nine days before clinical jaundice in infectious hepatitis.

<u>Determination</u>	<u>Normal Range</u>	<u>Discussion of Variations in Disease States</u>
Urine Examination (cont.):		
(g) Occult Blood	Negative	If the hemoglobin is present as rbc microscopic examination is more sensitive. However, this test is also used to verify hemoglobinuria.
"Benzidine Test"		
"Tollidine Test"		
"Gualac Test"		

SPINAL FLUID EXAMINATION

Xanthochromia		A yellow color usually due to hemoglobin from destroyed rbc.
Turbidity		May be due to bacteria or polycythemia (pus).
Glucose	50-80 mg. %	Decreased in bacterial meningitis and hypoglycemia. Increased in hyperglycemia.
Chlorides	700-760 mg. % NaCl	Decreased in bacterial (especially tuberculous) meningitis.
Proteins	15-40 mg. %	The protein is mainly albumin. Increased in meningitis, polyneuritis and tumors of the C.N.S. It may be of importance to differentiate albumin from globulin. For this technique see reference texts.

SAMPLE REQUIRED FOR ANALYSIS

Determination	Serum needed ml.	Blood needed ml.	Anticoagulant
Acetone	1	5	none
Alcohol, ethyl	2*	5*	oxalate
Alcohol, methyl	4*	5*	oxalate
Amino Acid N	1	5	none
Amylase	4 (0.4)	10 (2.0)	none or oxalate
Ascorbic acid	5	15	none
Bilirubin	4	10	none
Bilirubin, micro	0.2	1-2	none
Bromide	4	10	none
Bromsulfalein	1	5	none
Calcium	5	10	none
Carbon Dioxide	2***	8***	oxalate
Carbon Monoxide	0.4*	5*	oxalate
Cephalin Flocculation	0.4	5	none
Chloride	0.4	5	none
Cholesterol	4 (0.2)	10 (1)	none
Congo Red	2	5	none
Creatinine	4 (7)	10 (15)	none
Esterase	4	10	none
Fibrinogen	3* (1)*	5*	oxalate
Glucose	2*	5*	oxalate
Icterus index	1	5	none
Iodine (protein bound)	2	5	none
Iron	8	20	none
Iron-binding capacity	12	25	none
Lipase	4	10	none
Phosphate, inorganic	2	5	none
Phosphatase	2 (0.2)	5 (1)	none
Potassium	1 (0.4)	5 (1)	none
Protein-total	1 (0.2)	5 (1)	none
A/G ratio	1	5	none
Prothrombin time	0.2**	4.5**	oxalate
Prothrombin consumption	0.2***	5***	none
Salicylates	1	5	none
Sodium	1 (0.4)	5 (1)	none
Sulfonamides	1*	5*	oxalate
Thiocyanates	2	5	none

Determination	Serum needed ml.	Blood needed ml.	Anticoagulant
Thymol turbidity	0.2	5 (1)	none
Transaminase	0.6	5	none
Urea	2* (0.4)	5* (1)	oxalate
Uric Acid	2	5	none
Zinc turbidity	0.4	5	none

The above volumes represent the volume of serum (or other fluid) needed for the actual analysis in duplicate; the volume of blood which is required for the production of that amount of serum with a margin of safety; and the type of anticoagulant required.

*Whole Blood

**Special oxalate required

***Special treatment required

CONTROL PROCEDURES IN THE CLINICAL CHEMISTRY LABORATORY

The clinical laboratory should provide itself with a system of control which will alert the analyst so that errors will be quickly detected. Some of these systems are listed below.

A. Analysis of known solutions:

In most of the methods listed, the use of standard solutions is part of the procedure outlined and is therefore an integral part of the system of control. Another part of this method, when the photometric technique is used, is the maintenance of suitable records, so that any untoward variations in the amount of color produced by the standard (and by the blanks) will at once cause an investigation of the cause.

B. Replicate determinations:

This is the only procedure which will allow an estimate of precision (see statistics, p. 317). In most cases the replication should start at the original source material so as to include all the possible variations in the measurements which are made. In most clinical chemistry laboratories, single analyses only are made. It would be better if all analyses were done at least in duplicate.

C. "Blind analysis:"

Solutions of pure compounds, whose composition is known only to the laboratory director and not to the analyst can be a very convenient method of control. If a number of such solutions are prepared, each suitable for several different determinations with different known amounts of substances present, this will prevent conscious or unconscious bias in the reported results.

D. Serum Pool Analysis:

The "known" solution may be a serum pool. A large volume of serum may be pooled, divided into convenient portions and frozen. Then, each day or week, one sample may be thawed and analyzed. This method is limited to the control determinations of those substances not altered by this freezing technique. Commercial preparations are available which have been dried from such a frozen state and when reconstituted with water, serve as a very convenient standard serum pool. A number of such pools may be collected, analyzed and used for control purposes as outlined under C above.

E. Laboratory inter-comparison:

It is extremely difficult to make comparisons between laboratories due to the use of different procedures and due to the difficulty of transporting specimens without chemical alterations. This, is, however, potentially a very important method of control. It is especially convenient for two or three laboratories to serve as a continual check on each other by a system of continuous sample exchange.

F. Range of variation:

The person writing reports and checking results should be familiar with the usual range of values obtained in each test, not only for normal cases, but also in various pathological variations. Whenever the results are outside the realm of possibility or even probability, the results should be rechecked. In this judgment process, the diagnosis is important, so the request card should be consulted (even though frequently its information will be inadequate). Also, the results of other tests on the same patient bearing on the same problem will help.

1. Normal Ranges

Most laboratories maintain a list of normal values. These are usually expressed as a range. Values outside this range are regarded as abnormal or at least with suspicion that they may be abnormal. In order to more clearly comprehend what is meant by "variation" and "normal range" we list the types of variability encountered in the field of clinical chemistry.

a. Analytical variation. This is the range of values to be expected if a large number of identical samples of the same serum are carried through the analytical steps involved.

b. Individual variation. This is the range of values to be expected if the serum (for example) of a large number of different persons is analyzed. The samples are taken at comparable times to minimize the effect of certain variables mentioned in the next paragraph.

c. Physiological variation: This is the range of values to be expected if samples are taken from one individual at various times during the day or night; at various times during his life; or under various types and degrees of physiological stress. Some of these variations are predictable or controllable; others are uncontrollable. They are due to such variables as: age, sex, previous dietary history, body weight and height, mental state, degree of muscle activity, etc.

To minimize some of these variations blood samples are usually taken with the patient in a "fasting" or "post-prandial" state, some 12-14 hours after the last meal. In some cases it is important to avoid exercise previous to blood collection, or during a series of blood collections.

Few of the determinations in this manual have been studied sufficiently to obtain a complete knowledge of the effect of all of the factors listed above. In most of them, the analytical precision can be stated, and in most, the individual variation of the fasting samples is known but the physiological variation is for the most part still incompletely studied.

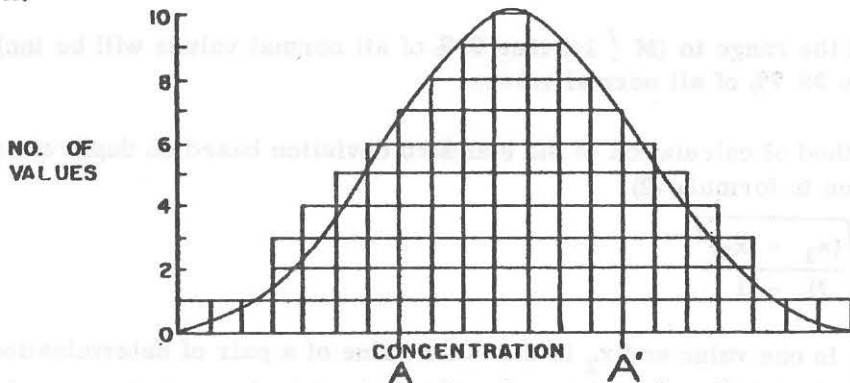
STATISTICS

Statistical analysis is a vast and growing field with many facets. It will suffice for our purposes to outline very simple concepts of two properties of collected data. These two properties may be termed measures of

- a. The central tendency
- b. The dispersion or the "spread" of the data.

The average or arithmetic mean will be used to express the central tendency. This is merely the sum of all the values determined, divided by the number of the determinations.

The tendency to dispersion is less easily defined. In any large series of analyses it will be noted that the data may be expressed somewhat as shown in the following bar graph.



The bar graph shows the actual results of a series of analyses, on normal serum, while the smooth solid curve is a theoretical curve based on an infinite number of analyses.

Dispersion of the results obtained in a series of analyses (say on normal serum) can be expressed as a range from the lowest to the highest values. This is perhaps the most common method used in clinical pathology today. It is better, however, to calculate the standard deviation by formulae (1) or (2).

The standard deviation is a mathematical measure of the dispersion or "spread" of the data to either side of the mean or average value. It may be calculated using formula (1).

$$(1) \quad \sigma = \sqrt{\frac{\sum (\bar{x} - x)^2}{N - 1}}$$

\bar{x} = arithmetic mean of N determinations

x = value of a single determination

σ = standard deviation

$\bar{x} - x$ = deviation of a single determination from the mean

Σ = "sum of"

M = mean or average

Thus, after a method is well-known and the mean and the standard deviation have been established, a value which deviates from the mean by more than 3 times the standard deviation (3σ) can be considered "abnormal" or "not due to random errors" since only 3 times in a thousand (0.3%) will the deviation exceed 3σ by mere chance.

When this is done, we can say with considerable certainty that 68% of all normal values will fall in the range between the mean minus one standard deviation and the mean plus one standard deviation ($M \pm 1\sigma$); that is to say, that the range A to A' in the figure includes about 68% of the area under the distribution curve.

If we extend the range to ($M \pm 2\sigma$) then 95% of all normal values will be included; ($M \pm 3\sigma$) includes 99.7% of all normal values.

Another method of calculation of the standard deviation based on duplicate determinations is given in formula (2).

$$(2) \quad \sigma = \sqrt{\frac{(x_1 - x_2)^2}{N - 1}}$$

where x_1 is one value and x_2 is the other value of a pair of determinations on the same sample.

Example: Two different technicians analyzed the same series of serums for total cholesterol and collected the following data.

Serum #	Cholesterol Value #1	(mg. %) Value #2	(1 - 2)	(1 - 2) ²	
32	97	95	2	4	
33	104	108	4	16	
35	111	108	3	9	
39	104	108	4	16	
40	135	123	12	144	
41	111	108	3	9	The data are
42	111	95	16	256	analyzed
48	97	95	2	4	
50	118	108	10	100	
53	104	101	3	9	using formula
54	118	123	5	25	
58	111	115	4	16	(2)
59	97	101	4	16	
70	97	115	18	324	
73	126	123	3	9	
75	126	129	3	9	
880	135	126	9	81	
885	126	118	8	64	
887	135	117	18	324	
				1435	$= \sum (x_1 - x_2)^2$
				18	$= N-1$

$$\sigma = \sqrt{\frac{1435}{18}} = \sqrt{79.7} = 8.92$$

The standard deviation is thus 8.92 mg. cholesterol per 100 ml. serum. A difference, then, of more than 26.8 mg. (3×8.92) between two analyses on the same serum would occur only 3 times out of a thousand analyses and thus probably represents something other than random error.

This same technic applied to serum samples from a number of normal human beings would allow us to state that a variation from the mean or average normal value which exceeds more than 3 times the standard deviation represents more than random variation and may be due to physiological or pathological variation in the patient.

Sometimes it is convenient to express the standard deviation in terms of "% of the mean." It is then called the "coefficient of variation."

Example: If a series of determinations gave a mean and standard deviation of 124 ± 8.9 , then the coefficient of variation would be

$$\frac{8.9}{124} \times 100 = 7.2\%$$

$$\frac{8.5}{100} \times 100 = 8.5\%$$

Example: If a series of determinations gave a mean and standard deviation of 104 ± 8.5, then the coefficient of variation would be

the mean. It is then called the "coefficient of variation."

Random variation and may be due to physiological or pathological variation in the patient.

This same technique applied to serum samples from a number of normal persons would allow us to state that a variation from the mean or average normal value which exceeds more than 3 times the standard deviation represents more than 99.7% of the total population.

$$S = \sqrt{\frac{\sum (x - \bar{x})^2}{n}} = \sqrt{\frac{18}{10}} = 1.34$$

Sum	Cholesterol	log R	(1 - S)	(1 + S)
987	130	117	18	22
880	125	120	2	8
75	120	120	2	8
70	120	120	2	8
68	115	115	4	10
58	111	111	4	10
54	118	123	2	8
53	104	101	2	8
50	110	108	10	10
48	97	97	2	8
45	111	95	16	20
41	117	108	0	0
40	105	122	12	16
39	104	108	1	5
38	111	108	3	7
37	104	108	4	8
32	97	97	2	8

The data are

not given

using formula

(S)

$\sum (x - \bar{x})^2 = 18$

$S = \sqrt{\frac{18}{10}} = 1.34$

$1 - S = 0.66$

ACID BASE EQUILIBRIUM AND WATER BALANCE

The cations (or "base") Na^+ and K^+ , and the anions Cl^- and HCO_3^- play an important role, by means of their effective osmotic pressure, in the regulation of the exchange of water between the cells of the body and the plasma and interstitial fluid. They are also concerned with the acid-base equilibrium of the body.

It is impossible to discuss the changes in any one of these ions without discussing the others because of the close interplay between and among them. As a whole, the body strives to maintain (a) a constant electrolyte composition (especially in regard to total osmotic pressure, which is almost equivalent to saying total electrolyte concentration) and (b) a constant pH.

The body is continually manufacturing "volatile" acid carbon dioxide (H_2CO_3), which is excreted largely through the lungs. In smaller quantities, it forms HCl , H_2SO_4 , H_3PO_4 and others. Most of these latter are non-volatile and must be excreted by the kidney. It is the momentous problem of the lungs and kidneys selectively and judiciously to excrete the correct substances in the right amounts, so as to maintain the two previously mentioned important quantities, (a) osmotic pressure or electrolyte concentration and (b) pH.

The pH depends on the ratio of $\text{HCO}_3^-/\text{H}_2\text{CO}_3$ and changes in the acid base equilibria of the body are quickly reflected in the HCO_3^- of the plasma. The H_2CO_3 of the plasma will rise and fall with the HCO_3^- of the plasma and the ratio $\text{HCO}_3^-/\text{H}_2\text{CO}_3$ will be very close to 20/1; this control is maintained by variation in the depth and rapidity of respiration and illustrates the strong attempt by the body to maintain a constant pH. HCO_3^- then is the most labile or changeable of the electrolyte components and any change in the others will be reflected in a change in HCO_3^- . Thus:

1. If Na^+ is decreased; HCO_3^- is decreased.
2. If Cl^- is decreased; HCO_3^- is increased.
3. If Cl^- is increased; HCO_3^- is decreased.
4. If non-volatile anions such as SO_4^{2-} , PO_4^{3-} (renal failure) or acetone body acids such as aceto-acetate or B-OH butyrate (diabetes) are increased, then HCO_3^- becomes decreased.

The above relationships of course depend on two axioms:

1. The total cation concentration must be equal to the total anion concentration.
2. In general, the total electrolyte concentration is kept relatively constant.

The inter-relationships may be seen more clearly by a few examples, keeping in mind that the kidney also operates in the regulation of acid-base equilibrium by (1) excreting an acid urine, and (2) synthesizing ammonia to replace Na^+ as a cation. Both of these methods conserve body base (Na^+). In renal failure inadequacy of these "base-conservation" mechanisms causes a loss of Na^+ into the urine and a decrease in plasma Na^+ concentration.

Severe loss of plasma volume also may cause a secondary type of renal insufficiency or failure with similar results.

EXAMPLES OF ELECTROLYTE CHANGES IN DISEASE

	Normal values	meq./liter	Average
Cations	Na	137-147	142
	K	4.1 - 5.6	5
	Ca	4.1 - 5.6	5

Anions	HCO ₃ ⁻	23 - 31	27
	Cl ⁻	96 - 105	103

- I. Severe Extracellular Fluid Loss "Dehydration": This may be caused by inability to eat or deprivation of water and food. No changes are seen in the concentration of electrolytes at first. The plasma volume decreases, and the electrolyte components of plasma are excreted in the urine proportionately to the plasma volume decrease. When the plasma volume has decreased so that renal failure occurs, loss of Na⁺ increases, serum Na⁺ decreases, non-volatile anions increase and HCO₃⁻ decreases secondarily to the changes in Na⁺ and non-volatile anions.
- II. Vomiting (Severe): Decreased plasma Cl⁻ due to loss of Cl⁻ in HCl of gastric juice. There is a decrease in sodium and increase in non-volatile anions because of decreased renal function secondary to reduced plasma volume. (In mild vomiting only a decrease in Cl⁻ and an increase in HCO₃⁻ may be seen.)
- III. Chronic Nephritis (Advanced): The kidney's reduced ability to excrete fixed non-volatile anions results in
- An increase of these anions in the plasma.
 - A decreased plasma HCO₃⁻.
 - The reduced ability to conserve base results in a reduced serum Na⁺.
- IV. Diarrhea: There is a decrease in Na⁺ because of loss of secretion high in Na⁺ content. A further decrease in Na⁺, and an increase in non-volatile anions and thus a decrease in HCO₃⁻ is seen in more severe diarrhea.
- V. Addison's Disease:
- Primary defect: a deficit of adrenal cortical hormones results in urinary loss of Na⁺ and retention of K⁺.
 - Results in
 - Decreased serum Na⁺ (and decreased plasma volume).
 - Decreased HCO₃⁻ (parallels Na⁺ decrease).
- VI. Diabetes:
- Defective metabolism of carbohydrates which results in an excessive catabolism of fats and a hyperglycemia.
 - The by-products of fatty acid metabolism, B-OH butyric acid, and acetoacetic acids are strong acids which require the excretion of much Na⁺ and

result in a lowered plasma Na^+ . Also glucose is excreted in the urine, requiring the excretion of water and some Na^+ thus further lowering plasma Na^+ .

- C. Plasma Na^+ has decreased, non-volatile anions have increased; therefore, HCO_3^- decreases to maintain anion-cation equivalency. Since H_2CO_3 must then be reduced to maintain the pH constant, hyperpnea is clinically observed.
- D. As the "dehydration" proceeds due to loss of water along with glucose, and with the excretion of the sodium salts, renal dysfunction occurs which results in still further loss of Na^+ , and further increase in plasma non-volatile anions.
- E. This is a "vicious cycle" and finally the pH is lowered, and the plasma volume may be lowered enough to cause "shock" or peripheral vascular collapse with a low blood pressure.

Laboratory Tests:

- Blood - Hgb & hematocrit - both high - suggesting decreased plasma volume - hemoconcentration.
- Plasma - Na^+ - low
 HCO_3^- - low
 Cl^- - normal or low
pH - low
- Urine - Sugar - positive - glycosuria
Acetone bodies - positive

During and after vigorous treatment of diabetic coma, potassium is rapidly returned to the cells from which it was drawn or lost during the acidosis. Therefore it is important to replace K^+ and to control its use therapeutically by plasma K^+ determinations.

Following are the normal values and conversion factors for the various ions in serum:

	Conversion Factor	Normal Range	Average
Sodium	$\frac{(\text{mg.}/100 \text{ ml.}) \times 10}{23} = \text{mEq. Na}^+/\text{l.}$	137-147 mEq./L	142
Potassium -	$\frac{(\text{mg.}/100 \text{ ml.}) \times 10}{39} = \text{mEq. K}^+/\text{l.}$	4.1 - 5.6 "	5
Calcium -	$\frac{(\text{mg.}/100 \text{ ml.}) \times 2 \times 10}{40} = \text{mEq. Ca}^{++}/\text{l.}$	4.1 - 5.6 "	5
Bicarbonate -	$\frac{\text{volumes } \%}{2.24} = \text{mEq. HCO}_3^-/\text{l.}$	23 - 31 "	27
Chloride - (expressed as mgm. of NaCl/100 ml.)	$\frac{(\text{mg.}/100 \text{ ml.}) \times 10}{58.45} = \text{mEq. Cl}^-/\text{l.}$	96 - 105 "	103
Protein (anion)	$(\text{g.}/100 \text{ ml.}) \times 2.43 = \text{mEq. Prot.}^-/\text{l.}$	15-19 "	17
PO_4^{1-8}	$(\text{mg.}/100 \text{ ml.}) \times 0.580 = \text{mEq. PO}_4^{1-8}/\text{l.}$	1.7 - 2.9	2.3

COAGULATION OF BLOOD

In order to understand (1) the part prothrombin plays in the coagulation of blood, (2) its importance to the practice of clinical medicine, and (3) the importance of the various factors and reagents involved in the laboratory determination, an abbreviated explanation of the clotting mechanism will be presented.

The processes involved in the coagulation of blood may be divided into three steps (for purposes of discussion). These are: (1) Activation of thromboplastin, (2) Conversion of prothrombin to thrombin, (3) Conversion of fibrinogen to fibrin.

Stage 1: Activation of thromboplastin. A number of factors are important in this activation:

- A. Platelet factors
- B. Anti-hemophilic factors
 - i. Anti-hemophilic globulin (AHG) in plasma, not present in aged serum, not adsorbed by BaSO_4 .
 - ii. Plasma thromboplastin component (PTC) ("Christmas factor") present in serum, adsorbed by BaSO_4 .
 - iii. Plasma thromboplastin antecedent (PTA), present in aged serum, not adsorbed by BaSO_4 .
- C. "Contact factor"---wetttable surfaces increase rate of formation of thromboplastin.
- D. "Tissue factor"---tissue extracts increase rate of formation of thromboplastin.
- E. Ca^{++} is necessary for thromboplastin formation.

There is disagreement as to the actual source of the thromboplastin. It may be present in plasma as an inactive precursor (Quick's thromboplastinogen), and merely activated by the tissue and platelet factors. Others believe the reverse is true, that the inactive precursors are present in tissue or in platelets.

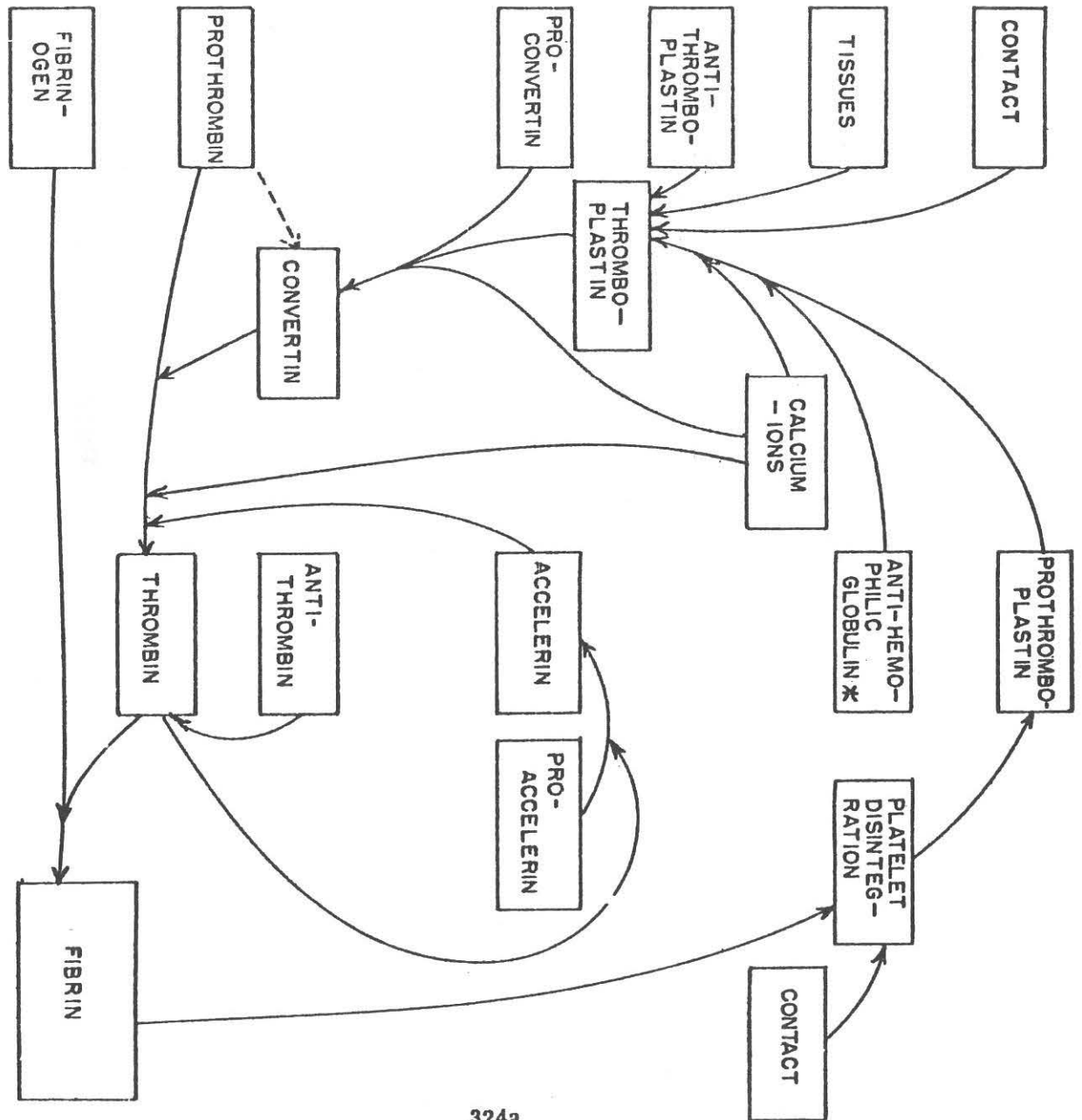
Stage 2: Conversion of prothrombin to thrombin. Again a number of different factors are important in this conversion.

- A. Thromboplastin - derived from Stage 1.
- B. Ca^{++} is also necessary in this stage.
- C. Prothrombin is present in plasma and is converted to thrombin. It is relatively stable, and is adsorbed by BaSO_4 .
- D. Accessory factors
 - i. Proconvertin (plasma) or convertin (serum) is required for the conversion of prothrombin to thrombin (physiologically). It is stable, and is adsorbed (as is prothrombin) by BaSO_4 .
 - ii. Proaccelerin (plasma) or accelerin (serum) merely accelerates the conversion of prothrombin to thrombin, (i.e., is not absolutely required) in the physiological system. It is quite labile and disappears rapidly from 37°C . incubated plasma, it is not adsorbed by BaSO_4 (differing from proconvertin and prothrombin).

OWREN'S (1953) THEORY OF BLOOD COAGULATION

Reference: American Journal of Medicine 14, 201 (1953).

This schema represents the inter-relationships between the various stages of coagulation. It should be noted that the first stage of coagulation is under intensive investigation now and at least two more factors, present in plasma have been shown to be necessary for the rapid formation of thromboplastin in blood. These are plasma thromboplastin component (PTC)* the "Christmas" factor; and plasma thromboplastin antecedent (PTA)*. There may be more.



Stage 3: Conversion of fibrinogen to fibrin (clot).

A. Thrombin--produced in Stage 2.

B. Fibrinogen--needed as substrate to the thrombin action. No other factors are required.

The characteristic which above all else has been most intriguing to investigators of coagulation is the suddenness of the clotting once it started to occur. This rapidity is due to what electronic engineers call "positive feed-back", or what biologists call "auto-catalysis", that is, in the various stages of coagulation, certain products stimulate or accelerate the primary reactions, thus, further accelerating the overall process. This can be seen by a view of Owren's (1953) schema seen on the facing page, American J. Med. XIV, 201 (1953).

In addition to these "positive feed-back" systems there also are some "negative feed-back" mechanisms or inhibitors system.

- A. Accelerin \longrightarrow inactive accelerin. (catalyzed by thrombin)
- B. Thrombin / Anti-thrombin \longrightarrow inactive thrombin.
- C. Profibrinolysin \longrightarrow fibrinolysin (catalyzed by fibrinokinase)
- D. Fibrin \longrightarrow fibrin split products (catalyzed by fibrinolysin)
- E. Thromboplastins are neutralized by anti-thromboplastins.
- F. Fibrinolysin is neutralized by anti-fibrinolysin.

Synonymy: Since there have been many different workers in the field, there have been many terms invented which apply to the same factor. Some of these which may cause confusion are listed here:

1. Pro-accelerin (Plasma) and accelerin (serum) Factor V. (Owren)
 - Ac (Accelerator) globulin
 - Prothrombin accelerator
 - Plasmatic cofactor
 - Labile factor - Quick
 - Prothrombin A Quick--1943
 - Plasma prothrombin conversion factor (PPCF)
2. Pro-convertin (plasma) convertin (serum)
 - Serum prothrombin conversion accelerator (SPCA)
 - Stable Prothrombin conversion factor
 - Factor VII (Koller)
 - Stable Factor

COAGULATION DEFECTS

Stage 1: Defects

Hemophilia: A deficiency of anti-hemophilic globulin of plasma (AHG).

Thrombocytopenia: A deficiency of platelets and platelet factor.

"Christmas" disease: A deficiency of plasma thromboplastin component (PTC).

Named after the family in which this defect was first noted.

(PTA) deficiency: Described by Rosenthal (1955)*

* A New First-stage Clotting Component. "Plasma Thromboplastin Antecedent".

Rosenthal, Blood 10, 120 (1955).

Stage 2: Defects

Prothrombin deficiency: due to: (1) Vitamin K deficiency and gross liver disease.

Vitamin K is required by the liver before synthesis of prothrombin can be carried out. Since Vitamin K is fat-soluble, its absorption from the intestine is markedly reduced in the absence of biliary and pancreatic secretions. Gross liver disease such as cirrhosis and acute yellow atrophy and acute chloroform poisoning will interfere with the formation of prothrombin even in the presence of adequate dietary or intramuscular synthetic Vitamin K.

(2) Hemorrhagic disease of the new-born before the routine pre-natal use of Vitamin K was fairly common (1/400). Since the use of the Vitamin it is much less common, and thus apparently was due to prothrombin deficiency secondary to a Vitamin K deficiency which in turn was probably due to the absence of an adult type of microbial flora in the infant's intestine. In adults, much of the Vitamin K required is obtained through the kind services of intestinal bacteria. However, the exact coagulation defect in the new-born has not been investigated in the light of the newer Stage 2 factors.

(3) Dicoumarol poisoning: This chemical causes a clotting defect first noted in cattle feeding on spoiled sweet clover. It is due to a lack of prothrombin (and also pro-convertin). This drug and its chemical relatives are used widely for the treatment of patients with thrombosis or thrombotic tendencies.

(4) Congenital Prothrombin Deficiency: Though there have been alleged cases in which familial prothrombin deficiency was present, all but one of these probably were cases of proconvertin or pro-accelerin deficiency, see below. In any case, instances of congenital failure of the liver to manufacture prothrombin must be very rare, in the presence of adequate Vitamin K and normal liver functions.

Pro-convertin deficiency: (1) Vitamin K deficiency and/or obstructive jaundice and steatorrhea (poor absorption) cause some decrease in pro-convertin.

(2) Dicoumarol as used in treatment of thrombosis decreases pro-convertin as well as prothrombin.

(3) Congenital--there are at least six cases of a hemorrhagic tendency from early childhood which have been shown to be due to a pro-convertin deficiency.

(4) Tromexan treatment: It affects prothrombin very little and its main effect is to decrease pro-convertin.

Pro-accelerin deficiency: (1) Liver disease which affects the liver cells decreases pro-accelerin. Obstructive jaundice (uncomplicated) does not affect pro-accelerin.

(2) Post-operatively pro-accelerin decreases to a minimum at about the third day post-operatively and returns to normal on about the ninth day.

(3) Congenital and familial: a number of cases have been described, especially in patients being treated with anti-coagulants. When there is significant time lapse between collections and the prothrombin assay, there exists the possibility of a loss of pro-accelerin.

(4) Coagulation: The process of clotting apparently uses up pro-accelerin and in massive in vivo clotting pro-accelerin deficiency may occur.

Stage 3: Defects

Fibrinogen deficiency: Plasma fibrinogen must be below 100 mg.% to affect the prothrombin time. (1) Congenital--very rare: The patient's bleeding time may be nearly normal illustrating that mechanisms other than coagulation are important in control of slight hemorrhage.

(2) Massive in vivo clotting may cause rapid defibrinogenation of the blood.

Other Coagulation Defects:

"Circulating anti-coagulants" may interfere with thromboplastin or prothrombin or with thrombin activity. Some (heparin) have been shown to interfere with pro-accelerin. "Fibrinolysin" rapidly lyses clots and when present in vivo in large amounts may render the blood "incoagulable", e.g., in massive intravascular clotting such as post-partum uterine hemorrhage.

Anticoagulant Therapy:

Heparin Group: Heparin is a complex polysaccharide with many sulphuric acid groups. It is not effective by mouth but lengthens clotting time when given intravenously or intra-muscularly. Its duration of action is relatively short (4-6 hours) and is quite expensive. Its administration is controlled by estimation of whole blood clotting times. Heparin may affect thromboplastin generation, prothrombin conversion and pro-accelerin activity.

Dicoumarin Group: These drugs affect factors mainly in Stage 2, the prothrombin conversion to thrombin.

Dicoumarol: decreases prothrombin and pro-convertin; does not affect pro-accelerin except perhaps to increase its lability.

Tromexan: decreases pro-convertin, does not affect prothrombin. A short acting drug (12-24 hours).

Hedulin: Apparently affects only prothrombin, but is a shorter acting drug than dicoumarol.

References:

The Coagulation of Blood-Methods of Study by L.M. Tocantins, M.D., Green and Stratton, 1955.

Human Blood Coagulation. Biggs, and Macfarlane, Blackwell, 1953.

TURBIDITY STANDARDS

References:

1. Shank, R. E. and Hoaglund, C. L., J. Biol. Chem. 162, 133 (1946).
2. Ducci, H., J. Lab. & Clin. Med., 32, 1266 (1947)

Some of the methods described depend upon the development of a turbidity by the addition of a reagent to serum, urine or spinal fluid. The degree of turbidity can be estimated by the photometer. This is done in the methods described for thymol turbidity, gamma globulin, urine and spinal fluid protein. These methods are, in general, considerably less accurate than are good chemical methods.

Originally the unknowns were compared to gelatin standards whose turbidity was developed by adding formaldehyde.

Recently, the use of turbidity standards of barium sulphate suspensions has become popular. These are prepared as described below.

Reagents:

1. Barium chloride 0.0962 M (2%)
2. Sulfuric acid 0.2 N
3. Stock suspension

In a 100 ml. volumetric flask place 3.0 ml. of the 2% BaCl_2 solution. Cool to 0°C . in ice bath. Add 0.2 N H_2SO_4 at 0°C . with shaking up to the mark. Mix rapidly and completely. Do not attempt to store.

4. Dilute Standards -

20 unit standard

2.70 ml. suspension
0.30 ml. H_2SO_4
3.00 ml. total volume

10 unit standard

1.35 ml. suspension
1.65 ml. H_2SO_4
3.00 ml. total volume

Other dilutions may be used and the amounts may be varied to give suitable total volumes. Place the dilute standards in matched photometer cuvetts, read against distilled water as a blank, and plot D readings against turbidity units.

The data can then be used in the preparation of graphs other determinations in which a turbidity standard is used.

Results of turbidity comparisons made using these standards as given above will be expressed in MacLagan units. One MacLagan unit represents 10 mg. protein per 100 ml. of fluid (urine, spinal fluid, or thymol turbidity, etc.). Recently the A.A.C.C. (American Association of Clinical Chemists) published in their first volume of Standard Methods of Clinical Chemistry, directions for Shank and Hoagland BaSO_4 standards. The procedure, as suggested in SMCC, with any given serum results in twice the number of turbidity units as would be the correct number of MacLagan units. This should be considered in comparing results of one laboratory with another.

CALIBRATION OF PHOTOMETER TUBES

In order to be able to compare the optical densities of standards, unknowns and blanks, it is necessary that the nominal optical thickness of the cuvetts or test tubes which are used be known or be identical. There are two ways in which to insure this.

1. All readings in the photometer are made using the same photometer tube, rinsing with solution each time, and being sure that the tube is oriented identically and is clean and dry on the outside at each reading. The cuvet may be fixed in position and the solution removed by a drain or by suction.

2. When it is impossible or inconvenient to use Method 1, we must resort to the selection of a number of tubes which will give the same effective optical depth when used in the photometer.

Apparatus:

Photometer using wavelength 515 m μ .

A stock of test tubes of suitable dimensions.

The number of test tubes required will usually be about two to three times the number of selected tubes finally needed. This number applies only to original selection of one homogeneous group from stock supply, since rejects from one group may fit into another later group.

Reagents:

1. Stock solution: Weigh out accurately 0.2000 g. KmnO_4 and transfer quantitatively to a one-liter volumetric flask. Add 1.0 g. KIO_4 and 50 ml. syrupy phosphoric acid (85-90%). Dissolve completely in distilled water, dilute to one liter, and mix well.

2. Diluting solution. Dissolve 1.0 g. of KIO_4 in water, add 50 ml. of syrupy phosphoric acid (85-90%) dilute to one liter and mix well.

3. Working solution for "1 cm." tubes. Place in a one-liter volumetric flask 150 ml. of stock solution, dilute to mark with diluting solution, and mix.

4. Working solution for large photometer tubes ("2 cm."). Place in a one-liter volumetric flask 100 ml. of stock solution, dilute to mark with diluting solution, and mix. This solution was developed by Professor R. H. Hamilton of Temple University Medical School, Philadelphia, Pa.

Procedure:

1. Clean the test tubes well by cleaning fluid or detergent and rinse well with tap water and then well with distilled water.
2. Dry in oven (or overnight), inverted to avoid dust.
3. Fill 2/3 full of pure distilled water.
4. Allow photometer to warm up well—one to two hours. Then without inserting a photometer tube, set the galvanometer at 50.0 (called a center setting) or at some reading which, with a tube of distilled water inserted will give a reading of 90 to 95. Then all the tubes of water are read, checking each time to keep the ϕ at the predetermined value (e.g., 50.0). Note: If dove-tailing of the selected tubes with a larger set or with other sets is desired it will be necessary to choose one selected tube with a certain reading when filled with distilled water (e.g., 95% transmittance) and reserve this tube as a primary standard, protecting it from scratches and dirt, against which all the tubes may be compared.

Each tube before being read should be inspected for the following important factors:

- a. Cleanliness—even a finger print may seriously affect the reading.
 - b. Air bubbles
 - c. Orientation in the cuvet holder—the pyrex trademark is a convenient point to use. Also if the tube is too large and tends to stick, or too loose and tends to wobble, it should be discarded.
 - d. Striae—any tubes with striae should be discarded.
5. Record all readings on paper, keeping tubes in order. It may be more convenient in selecting a short series to record the galvanometer reading on the tube itself.
6. Discard from the series all tubes falling outside the predetermined limits. At the high end of the galvanometer scale this should be not greater than plus or minus 0.2 galvanometer units. For example, if the readings cluster about 94.7, the mode of the distribution is 94.7; then, all tubes with readings less than 94.5 and greater than 94.9 would be discarded.

7. Pour out the distilled water, dry as before and rinse with and fill 2/3 full of the permanganate solution.

8. Keeping the same ϕ setting, record the transmissions of each tube as before. The readings now will be between 30 and 40% transmittance.

9. Again discard all tubes varying more than the preselected limits from the mode. For example if we decide to allow a variability of plus or minus 0.1 galvanometer units and if the mode is 35.6, then all tubes with readings less than 35.5 and greater than 35.7 would be discarded.

10. Let us calculate what this means in terms of inaccuracy contributed to the determination by the phototubes. In order to calculate the optical densities we must correct for the fact that the blank tube was not set on 100 but on 94.7 (the mode of the distribution). $94.7/35.6 = 100/(37.6)$. (This is merely the calculation of the per cent transmittance of the permanganate solution.) Now the optical density corresponding to a

transmittance of 37.6 is 0.425 and the tolerances allowed above represent optical density variations of 0.001 and 0.002 for the high and the low ends of the scale respectively. If these variations are additive they would represent a 0.7% error in the final optical density as calculated. If greater error can be tolerated, then wider tolerance limits can be set up.

11. Precision bore tubing both cylindrical and square has recently become available and this may help to solve this problem of calibration. All of this tubing, however, while of constant internal thickness, has walls which vary in thickness and this impairs their optical properties somewhat.

VOLUME CALIBRATION OF TEST TUBES

In many determinations it is advantageous to dilute a solution to a given volume in a test tube. The test tubes may be calibrated by the following procedure:

Arrange test tubes in a rack so that they can be easily handled. They need not be perfectly clean but must be dry. Using a volumetric transfer pipet, transfer quantitatively the required volume of fluid such as 10 ml., 15 ml., etc.

Arrange the tube in an apparatus, so that the tube can be held vertically and at the same time rotated about its long axis.

Sighting across the lowest point of the meniscus (horizontally to avoid parallax) scratch opposite sides of the tube with a fixed diamond pencil. Recheck the accuracy of these points visually and then circumscribe the tube with the diamond point. Tubes may be numbered if required.

THE USE OF THE SLIDE RULE

This section is not intended to substitute for the excellent manuals of instruction which accompany most slide-rules. It is intended as a very brief statement regarding their use. Only persistent use of a slide rule will permit the owner to achieve that degree of familiarity which will serve to shorten the labor of mathematical calculations.

Multiplication:

The scale labeled C (on the slide) and the scale D (on the rule) are used for multiplication. These two scales are exactly alike. The total length of these scales has been separated into smaller parts by graduations. Note that the distance between 1 and 2 and between 2 and 3 etc., is not the same. It decreases as the number increases. It is carefully measured to decrease logarithmically. So that what we do in multiplication is to add lengths of the rule corresponding to the logarithm of the numbers we wish to multiply together.

Remembering that the sum of the logarithm of A and B equals the logarithm of the product $A \times B$

$$\log A + \log B = \log (A \times B)$$

We see that by adding lengths of rule we can multiply.

Similarly, by subtraction of lengths of the rule, we can divide

$$\log A - \log B = \log (A/B)$$

Example: Examples of simple multiplication and division will be given and your technique can be checked at any time by redoing these examples.

Multiplication: Multiply 2×3

1. Setting the scales

Set the left index (1) of the C scale on 2 of the D scale. Now find 3 on the C scale and read the product 6 on the D scale. Thus, the length for 2 plus the length for 3 will be the length for the product (6).

Division: Divide $8 \div 4$

1. Find 8 on the D scale, set 4 on the C scale over it and read the result 2 on the D scale under the index 1 of the C scale. The length for 8 minus the length for 4 is the length of the dividend (2).

Decimal Point Location:

The slide rule does not locate the decimal point. However in most cases the location of the decimal point can be estimated by "common" sense. Thus $122.1 \div 9.6$ is about $100/10$ and the answer is about 10 (12.73; not 1.273; not 127.3).

Further details and short-cuts can be seen and learned by reference to slide rule manuals.

Common Logarithms of Numbers

N	0	1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396
N	0	1	2	3	4	5	6	7	8	9

Common Logarithms of Numbers

N	0	1	2	3	4	5	6	7	8	9
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996
N	0	1	2	3	4	5	6	7	8	9

Optical Density (D) table for values of per cent light transmitted (T)

$$D = 2 - \log_{10} T, \text{ where } T = \frac{I}{I_0} \times 100$$

T	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0		3.000	2.699	2.523	2.398	2.301	2.222	2.155	2.097	2.046
1	2.000	1.959	1.921	1.886	1.854	1.824	1.796	1.770	1.745	1.721
2	1.699	1.678	1.658	1.638	1.620	1.585	1.569	1.569	1.553	1.538
3	1.523	1.509	1.495	1.481	1.469	1.456	1.444	1.432	1.420	1.409
4	1.398	1.387	1.377	1.367	1.357	1.347	1.337	1.328	1.319	1.310
5	1.301	1.292	1.284	1.276	1.268	1.260	1.252	1.244	1.237	1.229
6	1.222	1.215	1.208	1.201	1.194	1.187	1.180	1.174	1.167	1.161
7	1.155	1.149	1.143	1.137	1.131	1.125	1.119	1.114	1.108	1.102
8	1.197	1.092	1.086	1.081	1.076	1.071	1.066	1.060	1.056	1.051
9	1.046	1.041	1.036	1.032	1.027	1.022	1.018	1.013	1.009	1.004
10	1.000	0.996	0.991	0.987	0.983	0.979	0.975	0.971	0.967	0.963

T	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
10	1.000	996	991	987	983	979	975	971	967	963
11	959	955	951	947	943	939	936	932	928	924
12	921	917	914	910	907	903	900	896	893	889
13	886	883	879	876	873	870	866	863	860	857
14	854	851	848	845	842	839	836	833	830	827
15	824	821	818	815	812	810	807	804	801	799
16	796	793	790	788	785	783	780	777	775	772
17	770	767	764	762	759	757	754	752	750	747
18	745	742	740	738	735	733	730	728	726	724
19	721	719	717	714	712	710	708	706	703	701
20	699	697	695	693	690	688	686	684	682	680
21	678	676	674	672	670	668	666	664	662	660
22	658	656	654	652	650	648	646	644	642	640
23	638	636	635	633	631	629	627	625	623	622
24	620	618	616	614	613	611	609	607	606	604

T	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
25	602	600	599	597	595	593	592	590	588	587
26	585	583	582	580	578	577	575	573	572	570
27	569	567	565	564	562	561	559	558	556	554
28	553	551	550	548	547	545	544	542	541	539
29	538	536	535	533	532	530	529	527	526	524
30	523	521	520	519	517	516	514	513	511	510
31	509	507	506	504	503	502	500	499	498	496
32	495	493	492	491	489	488	487	485	484	483
33	481	480	479	478	476	475	474	472	471	470
34	469	467	466	465	463	462	461	460	458	457
35	456	455	453	452	451	450	449	447	446	445
36	444	442	441	440	439	438	437	435	434	433
37	432	431	429	428	427	426	425	424	423	421
38	420	419	418	417	416	415	413	412	411	410
39	409	408	407	406	405	403	402	401	400	399
40	398	397	396	395	394	393	391	390	389	388
41	387	386	385	384	383	382	381	380	379	378
42	377	376	375	374	373	372	371	370	369	368
43	367	366	365	364	363	362	361	360	359	358
44	357	356	355	354	353	352	351	350	349	348
45	347	346	345	344	343	342	341	340	339	338
46	337	336	335	334	333	333	332	331	330	329
47	328	327	326	325	324	323	322	321	321	320
48	319	318	317	316	315	314	313	312	312	311
49	310	309	308	307	306	305	305	304	303	302
50	301	300	299	298	298	297	296	295	294	293
51	292	292	291	290	289	288	287	287	286	285
52	284	283	282	281	281	280	279	278	277	277
53	276	275	274	273	272	272	271	270	269	268
54	268	267	266	265	264	264	263	262	261	260
55	260	259	258	257	256	256	255	254	253	253

T	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
56	252	251	250	249	249	248	247	246	246	246
57	244	243	243	242	241	240	240	239	238	237
58	237	236	235	234	234	233	232	231	231	230
59	229	228	228	227	226	225	225	224	223	223
60	222	221	220	220	219	218	218	217	216	215
61	215	214	213	213	212	211	210	210	209	208
62	208	207	206	206	205	204	203	203	202	201
63	201	200	199	199	198	197	197	196	195	194
64	194	193	192	192	191	190	190	189	188	188
65	187	186	186	185	184	184	183	182	182	181
66	180	180	179	178	178	177	177	176	175	175
67	174	173	173	172	171	171	170	169	169	168
68	167	167	166	166	165	164	164	163	162	162
69	161	161	160	159	159	158	157	157	156	156
70	155	154	154	153	152	152	151	151	150	149
71	149	148	148	147	146	146	145	144	144	143
72	143	142	141	141	140	140	139	138	138	137
73	137	136	135	135	134	134	133	133	132	131
74	131	130	130	129	128	128	127	127	126	126
75	125	124	124	123	123	122	121	121	120	120
76	119	119	118	117	117	116	116	115	115	114
77	114	113	112	112	111	111	110	110	109	108
78	108	107	107	106	106	105	105	104	103	103
79	102	102	101	101	100	100	099	099	098	097
80	097	096	096	095	095	094	094	093	093	092
81	092	091	090	090	089	089	088	088	087	087
82	086	086	085	085	084	084	083	082	082	081
83	081	080	080	079	079	078	078	077	077	076
84	076	075	075	074	074	073	073	072	072	071
85	071	070	070	069	069	068	068	067	067	066

T T	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
85	071	070	070	069	069	068	068	067	067	066
86	068	065	064	064	063	063	062	062	061	061
87	060	060	059	059	058	058	057	057	057	056
88	056	055	055	054	054	053	053	052	052	051
89	051	050	050	049	049	048	048	047	047	046
90	046	045	045	044	044	043	043	042	042	041
91	041	040	040	040	039	039	038	038	037	037
92	036	036	035	035	034	034	033	033	032	032
930	032	031	031	030	030	029	029	028	028	027
94	027	026	026	025	025	025	024	024	023	023
95	022	022	021	021	020	020	020	019	019	018
96	018	017	017	016	016	015	015	015	014	014
97	013	013	012	012	011	011	011	010	010	009
98	009	008	007	007	007	007	006	006	005	005
99	004	004	003	003	003	002	001	001	001	000

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LONG FORM OF THE PERIODIC TABLE

Period	IA	IIA											IIIB	IVB	VB	VIB	VII B	O
1	1 H 1.008																1 H 1.008	2 He 4.003
2	3 Li 6.940	4 Be 9.012											5 B 10.82	6 C 12.01	7 N 14.008	8 O 16.00	9 F 19.00	10 Ne 20.183
3	11 Na 22.997	12 Mg 24.32											13 Al 26.98	14 Si 28.09	15 P 30.975	16 S 32.065	17 Cl 35.457	18 Ar 39.944
4	19 K 39.100	20 Ca 40.08	21 Sc 44.96	22 Ti 47.88	23 V 50.94	24 Cr 52.01	25 Mn 54.93	26 Fe 55.85	27 Co 58.94	28 Ni 58.69	29 Cu 63.54	30 Zn 65.38	31 Ga 69.72	32 Ge 72.60	33 As 74.91	34 Se 78.96	35 Br 79.916	36 Kr 83.80
5	37 Rb 85.48	38 Sr 87.63	39 Y 88.92	40 Zr 91.22	41 Nb 92.91	42 Mo 95.95	43 Tc 98	44 Ru 101.7	45 Rh 102.91	46 Pd 106.7	47 Ag 107.880	48 Cd 112.41	49 In 114.76	50 Sn 118.70	51 Sb 121.76	52 Te 127.61	53 I 126.91	54 Xe 131.3
6	55 Cs 132.91	56 Ba 137.36	57-71 La-Lu Rare Earths	72 Hf 178.6	73 Ta 180.88	74 W 183.82	75 Re 186.31	76 Os 190.2	77 Ir 193.1	78 Pt 195.23	79 Au 197.2	80 Hg 200.61	81 Tl 204.39	82 Pb 207.21	83 Bi 209.00	84 Po 210	85 At 210	86 Rn 222
7	87 Fr 223	88 Ra 226.05		89-102 Actinides														
Rare Earths			57 La 138.92	58 Ce 140.13	59 Pr 140.92	60 Nd 144.27	61 Pm 145	62 Sm 150.43	63 Eu 152.0	64 Gd 156.9	65 Tb 159.2	66 Dy 162.46	67 Ho 164.94	68 Er 167.2	69 Tm 169.4	70 Yb 173.04	71 Lu 174.99	
Actinides			89 Ac 227	90 Th 232.12	91 Pa 231	92 U 238.07	93 Np 237	94 Pu 242	95 Am 243	96 Cm 243	97 Bk 245	98 Cf 246	99 Es 255	100 Fm 252	101 Md 256	102 No		

INTERNATIONAL ATOMIC WEIGHTS

1956

	Symbol	Atomic Number	Atomic Weight		Symbol	Atomic Number	Atomic Weight
Actinium	Ac	89	227	Mercury	Hg	80	200.61
Aluminum	Al	13	26.98	Molybdenum	Mo	42	95.95
Americium	Am	95	(243)*	Neodymium	Nd	60	144.27
Antimony	Sb	51	121.76	Neon	Ne	10	20.183
Argon	A	18	39.944	Neptunium	Np	93	(237)
Arsenic	As	33	74.91	Nickel	Ni	28	58.71
Astatine	At	95	(211)	Niobium	Nb	41	92.91
Barium	Ba	56	137.37	Nitrogen	N	7	14.008
Berkelium	Bk	97		Nobelium	No	102	(253)
Beryllium	Be	4	9.013	Osmium	Os	76	190.2
Bismuth	Bi	83	209.00	Oxygen	O	8	16
Boron	B	5	10.82	Palladium	Pd	46	106.7
Bromine	Br	35	79.916	Phosphorus	P	15	30.975
Cadmium	Cd	48	112.41	Platinum	Pt	78	195.09
Calcium	Ca	20	40.08	Plutonium	Pu	94	(242)
Californium	Cf	98		Polonium	Po	84	210
Carbon	C	6	12.011	Potassium	K	19	39.100
Cerium	Ce	58	140.13	Praseodymium	Pr	59	140.92
Cesium	Cs	55	132.91	Promethium	Pm	61	(145)
Chlorine	Cl	17	35.457	Protactinium	Pa	91	231
Chromium	Cr	24	52.01	Radium	Ra	88	226.05
Cobalt	Co	27	58.94	Radon	Rn	86	222
Copper	Cu	29	63.54	Rhenium	Re	75	186.22
Curium	Cm	96	(245)	Rhodium	Rh	45	102.91
Dysprosium	Dy	66	162.51	Rubidium	Rb	37	85.48
Einsteinium	E	99	(254)	Ruthenium	Ru	44	101.1
Erbium	Er	68	167.27	Samarium	Sm	62	150.35
Europium	Eu	63	152.0	Scandium	Sc	21	44.96
Fermium	Fm	100	(252)	Selenium	Se	34	78.96
Fluorine	F	9	19.00	Silicon	Si	14	28.09
Francium	Fr	87	(223)	Silver	Ag	47	107.880
Gadolinium	Gd	64	157.26	Sodium	Na	11	22.991
Gallium	Ga	31	69.72	Strontium	Sr	38	87.63
Germanium	Ge	32	72.60	Sulfur	S	16	32.066
Gold	Au	79	197.0	Tantalum	Ta	73	180.95
Hafnium	Hf	72	178.56	Technetium	Tc	43	(99)
Helium	He	2	4.003	Tellurium	Te	52	127.61
Holmium	Ho	67	164.94	Terbium	Tb	65	158.93
Hydrogen	H	1	1.0080	Thallium	Tl	81	204.39
Indium	In	49	114.76	Thorium	Th	90	232.05
Iodine	I	53	126.91	Thulium	Tm	69	168.94
Iridium	Ir	77	192.2	Tin	Sn	50	118.70
Iron	Fe	26	55.85	Titanium	Ti	22	47.90
Krypton	Kr	36	83.8	Tungsten	W	74	183.86
Lanthanum	La	57	138.92	Uranium	U	92	238.07
Lead	Pb	82	207.21	Vanadium	V	23	50.95
Lithium	Li	3	6.940	Xenon	Xe	54	131.30
Lutetium	Lu	71	174.99	Ytterbium	Yb	70	173.04
Magnesium	Mg	12	24.32	Yttrium	Y	39	88.92
Manganese	Mn	25	54.94	Zinc	Zn	30	65.38
Mendelevium	Mv	101	(256)	Zirconium	Zr	40	91.22

* A value given in brackets denotes the mass number of the isotope of longest known half-life.

/ Synthesized in 1957.

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